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(54) Title: BIOCATALYST INHIBITORS

(57) Abstract: The present invention concerns a method for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or beta-glucanolytic enzymes, inhibitors obtainable by said method, and process for obtaining micro-organism, plant or plant material wherein the activity of the inhibitor according to the invention is increased or reduced and to the use of the inhibitor, using the cited micro-organism, plant or plant material and/or the use of endoxylanases selected or modified using these inhibitors in a variety of process and applications.

BIOCATALYST INHIBITORS

Field of the invention

This invention relates to a method for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8)(also referred to as xylanase), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes, which are present in micro-organisms, plants, plant materials or fractions thereof, (such as cereals, cereal grains, cereal flours or fractions thereof). The method comprises the use of two or more enzymes, especially endoxylanases, during the screening for inhibition activity. In a preferred embodiment, one of the endoxylanases is from *Bacillus subtilis*.

This invention also relates to a method for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC:3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading

enzymes such as endoxylanase (such as EC: 3.2.1.8)(also referred to as xylanase), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes, which are present in micro-organisms, plants, plant materials or fractions thereof, (such as cereals, cereal grains, cereal flours or fractions thereof). The method comprises an affinity chromatographic step with immobilised enzymes, especially endoxylanases, and/or antibodies against the said inhibitors, especially antibodies against an endoxylanase inhibitor. In a preferred embodiment, the immobilised endoxylanases are those from *Bacillus subtilis* and/or *Aspergillus niger*.

The present invention is also related to inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes, obtainable by said methods, as well as to feed or food compositions comprising said inhibitors and to the use of said inhibitors for screening enzymes such as endoxylanases that are totally, more, less or not inhibited by said inhibitors or for modifying enzymes, such as endoxylanases in such way that they are totally, more, less or not inhibited by said inhibitors, as well as to the use of said inhibitors in different areas of food, feed and/or beverage technologies, such as malting and brewing, the production of

animal feedstuffs such as to increase their conversion, the production of refrigerated and/or frozen doughs, such as to reduce syruping, the production of baked and/or extruded cereal products such as straight dough, sponge and dough (all said dough compositions comprising flour and water) and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles, the production of starch derived syrups, sorbitol, xylose and/or xylitol, the wheat gluten-starch separation industry, maize processing, the improvement of plant disease resistance, in nutraceutical or pharmaceutical applications such as maintaining the structure of dietary fiber material, and in the field of paper and pulp technologies.

The present invention also relates to polynucleotide sequences encoding inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases), especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes and to said inhibitors obtainable by a recombinant production process using said polynucleotide sequences encoding the recombinant inhibitors. The invention also relates to feed or food compositions comprising said recombinant inhibitors and the use of said recombinant inhibitors for screening enzymes such as endoxylanases that are totally, more, less or not inhibited by said inhibitors or for modifying enzymes, such as endoxylanases in such way that they are totally, more, less or not inhibited by said inhibitors, as well as to the use of said recombinant inhibitors in different areas of

food, feed and/or beverage technologies, such as malting and brewing, the production of animal feedstuffs such as to increase their conversion, the production of refrigerated and/or frozen doughs, such as to reduce syruping, the production of baked and/or extruded cereal products such as straight dough, sponge and dough (all said dough compositions comprising flour and water) and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles, the production of starch derived syrups, sorbitol, xylose and/or xylitol, the wheat gluten-starch separation industry, maize processing, the improvement of plant disease resistance, in nutraceutical or pharmaceutical applications such as maintaining the structure of dietary fiber material, and in the field of paper and pulp technologies.

Background of the invention

Cereal grains contain three groups of important biopolymers: starch, proteins and non-starch polysaccharides. Starch and a large part of the protein fraction are located in the endosperm and serve as reserve material for the plant during germination and the initial stages of growth. They are degraded by amylases and proteases respectively [1]. The non-starch polysaccharides include mainly arabinoxylan (AX) and β-glucan which are part of the cell walls and are hydrolysed by xylanolytic and β-glucanolytic enzymes respectively [1, 2]. The degradation of these cell wall polysaccharides in the endosperm and aleurone layer during the germination improves the accessibility of starch and protein for amylases and proteases [3, 4]. Proteins that inhibit amylases [5-9] and proteases [10-13] have already been purified from cereals and have been characterised extensively. They possibly regulate the plant starch and nitrogen metabolism and/or play an important role in plant defence by inhibiting enzymic hydrolysis by micro-organisms and predators.

Recently, a new class of enzyme inhibitors, i.e. proteinaceous inhibitors of endo-β-1,4-xylanases (endoxylanases, EC: 3.2.1.8), has been discovered in cereals by Debyser and Delcour [14] and Debyser et al. [15]. Inhibition activity against such xylanolytic enzymes was found in different cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) [14, 16].

An endoxylanase inhibitor, named TAXI (*T. aestivum* L. endoxylanase inhibitor), was purified from wheat flour and characterised by Debyser and Delcour [14] and Debyser et al. [17] wherein for the screening of the inhibition activity a single endoxylanase from *Aspergillus niger* was used. TAXI has a molecular mass of ca. 40.0 kDa and occurs in two molecular forms A and B, B presumably as a result of proteolytic

modification of A [14, 16, 17]. As a result of reduction with β-mercaptoethanol, the modified molecular form B dissociates in two fragments with molecular masses of ca. 10.0 and 30.0 kDa respectively, whereas the molecular mass of the non-modified form does not change upon reduction. The inhibitor is heat sensitive and has a pI of ca. 8.8 [14, 16, 17]. Rouau and Surget [18] also found evidence for the presence of endoxylanase inhibitors in regular and durum wheats. These authors detected high inhibition activity against microbial endoxylanases in both wheat flour and bran. McLauchlan et al. [19] and Hessing and Happe [20] purified a wheat endoxylanase inhibitor structurally quite different from TAXI. In the isolation procedure [19], an endoxylanase, partially purified from a commercial *A. niger* hemicellulase preparation was used for the screening of the inhibition activity. The resulting inhibitor is monomeric, glycosylated and a heat sensitive protein. It has a pI of 8.7-8.9 [19] or higher than 9 [20], a molecular mass of 29.0 kDa [19] and 31.0 kDa [20] and was found to be a competitive inhibitor. The N-terminal amino acid sequence is 87 % identical with a sequence close to the N-terminus of the rice chitinase III polypeptide chain and shows no homology with the amino acid sequences of TAXI [19,20].

Further information on TAXI can be found in Sibbesen and Sørensen [36].

Summary of the invention

The present invention concerns methods for the separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes, preferably inhibitors of endoxylanase, of β-glucanase, of β-xylosidase, of α-L-arabinofuranosidase, and of other xylan, arabinoxylan and β-glucan degrading enzymes preferably obtained from micro-organisms, plants, plant materials or fractions thereof (such as cereals, cereal grains, cereal germs or fractions thereof, cereal flours or fractions thereof) by the use of two or more enzymes, especially endoxylanases, during the screening for endoxylanase inhibiting activity and/or by the application of affinity chromatography with immobilised enzymes, especially endoxylanases.

The inhibitory effect towards xylan and/or arabinoxylan hydrolysing enzymes can be e.g. demonstrated by the endoxylanase method with AZCL arabinoxylan (cfr. infra). Likewise, the inhibitory effect towards β-glucan hydrolysing enzymes can be e.g. demonstrated by the β-glucanase method with AZCL β-glucan (cfr. infra).

The present invention also concerns novel inhibitors obtainable by said purification methods, of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC:3.2.1.4), pentosanases and/or hemicellulases) especially inhibitors of pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes.

In this text, "An inhibitor of an enzyme" means

a molecule which is able to inhibit partially or totally the activity of said enzyme. In irreversible inhibition, the inhibitor is covalently linked to the enzyme or bound so tightly that its dissociation from the enzyme is very slow. In contrast, reversible inhibition may be characterised by a rapid equilibrium between the enzyme and the inhibitor. A competitive inhibitor blocks the active site and in this way prevents the substrate/active site interaction. As a consequence, the reaction rate is diminished. In the case of competitive inhibition, the inhibitor in many cases mimicks the normal substrate of said enzyme. For this type of inhibition, the Dixon plots (inverse of reaction rate, 1/V, versus inhibitor concentration, [I]) corresponding to the different substrate concentrations and the Lineweaver-Burk plots (inverse of reaction rate, 1/V, versus inverse of substrate concentration, 1/[S]) corresponding to different inhibitor concentrations intersect in the left quadrant and the vertical axis respectively. For non-competitive inhibition, both inhibitor and arabinoxylan can bind to the enzyme and this independent of the binding order. The inhibition can in most cases be explained by a change in conformation of the enzyme at or near the active site with a decreased turnover number as a result. In the case of non-competitive inhibition, however, the Dixon and Lineweaver-Burk plots intersect on the horizontal axis in the left quadrant. Competitive inhibition can be distinguished from non-competitive inhibition by determining whether the inhibition can be overcome by raising the substrate concentration. Inhibitors isolated from a specific biological species and that are of proteinaceous or glycoproteinaceous nature can be active against enzymes of the same species (i.e. endogenous enzymes) and/or against enzymes of different species (i.e. exogenous enzymes).

Advantageously, the inhibitors of the invention can be produced by micro-organisms or may be present in

various extraction media from micro-organisms or plant material, such as cereals or fractions thereof, such as cereal grains or fractions thereof, such as cereal germs or fractions thereof, such as cereal flours or fractions thereof, such as from wheat, durum wheat, rye, triticale, barley, sorghum, oats, maize and/or rice, from which they can be purified by the methods well known by the man skilled in the art. According to a preferred embodiment of the present invention, inhibitors are endoxylanase inhibitors which are typically water-soluble alkaline proteinaceous species, having a pI (i.e. -log of the isoelectric point) of greater than about 7.0. The endoxylanase inhibitor molecular weights as determined by SDS-page are typically 40-43 kDa. Following reduction with β-mercaptoethanol three SDS-page protein bands are found with SDS-page molecular weights of ca. 40-43 kDa, ca. 30 kDa, and ca. 10 kDa. The N-terminal sequences of the 40-43 kDa proteins or glycoproteins are typically as follows: SEQ ID No. 1 (TAXI I): Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Asp-Pro-Ala-Thr-Ser-Leu-Tyr-Thr-Ile-Pro-Phe-Xaa-Asp-Xaa-Ala, wherein the first Xaa being preferably Leu and wherein the second Xaa being preferably Leu; SEQ ID No. 2 (TAXI II): Lys-Gly-Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Asp-Thr-Ala-Thr-Ser-Leu-Tyr-Thr-Ile-Pro-Phe or SEQ ID No. 3 (HvXI): Lys-Ala-Leu-Pro-Val-Leu-Ala-Pro-Val-Thr-Lys-Asp-Ala-Ala-Thr-Ser-Leu-Tyr-Thr-Ile-Xaa-Xaa, wherein the first Xaa being preferably Pro and wherein the second Xaa being preferably Phe. The 30 kDa band has the above described typical N-terminal amino acid SEQ ID No.1 or SEQ ID No.2 or SEQ ID No.3, while the N-terminal amino acid sequence of the 10 kDa band is typically as follows: SEQ ID No. 4 (TAXI I): Gly-Ala-Pro-Val-Ala-Arg-Ala-Val-Glu-Ala-Val-Ala-Pro-Phe-Gly-Val-Xaa-Tyr-Asp-Thr, wherein Xaa being preferably Leu; or SEQ ID No. 5 (TAXI II): Gly-Ala-Pro-Val-Ala-Arg-Ala-Val-Ile-Pro-Val-Ala-Pro-Phe-Glu-Leu-Xaa-Tyr-Xaa-Thr-Lys-Ser-Leu-Gly-Asn, wherein the first Xaa being preferably Leu and wherein the second Xaa being

preferably Asp; or SEQ ID No. 6 (HvXI): Gly-Ala-Leu-Ala-Ala-Xaa-Gly-Val-Asn-Pro-Val-Ala-Pro-Phe-Gly-Xaa-Xaa-Tyr-Asp-Ala-Xaa-Thr-Xaa-Xaa, wherein the first Xaa is unknown, the second Xaa being preferably Leu, the third Xaa is unknown, the fourth Xaa is unknown, the fifth Xaa being preferably Asn, and wherein the sixth Xaa being preferably Gly.

Therefore, the present invention is also related to an inhibitor with a SDS-page molecular weight of typically 40-43 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 1. or SEQ ID No.2 or SEQ ID No.3

The present invention is furthermore also related to an inhibitor with a SDS-page molecular weight of typically 30 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 1. or SEQ ID No.2 or SEQ ID No.3.

The present invention is furthermore also related to an inhibitor with a SDS-page molecular weight of typically 10 kDa being a protein or glycoprotein having a marker whose amino acid sequence has more than 70% homology, preferably more than 85% homology, more preferably is identical with SEQ ID No. 4. or SEQ ID No.5 or SEQ ID No.6.

Advantageously, said markers are the end-terminal amino acid sequences of the protein or glycoprotein.

According to the invention, a marker of a protein or glycoprotein means a specific amino acid sequence (or its corresponding nucleotide sequence) that is able to distinguish one protein family from another protein family.

Another aspect of the invention involves the corresponding polynucleotide sequences of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes (sometimes also referred to as cellulases (EC: 3.2.1.4), pentosanases and/or hemicellulases) especially inhibitors of

pentosan degrading enzymes such as endoxylanase (such as EC: 3.2.1.8), β-xylosidase (such as EC: 3.2.1.37), and α-L-arabinofuranosidase (such as EC: 3.2.1.55), to inhibitors of cellulase (such as EC: 3.2.1.4), β-glucanase (such as EC: 3.2.1.73 or such as 3.2.1.6), and to inhibitors of other xylan, arabinoxylan and β-glucan degrading enzymes.

The invention concerns isolated polynucleotides that encode for endoxylanase inhibitors from wheat, rye, rice, maize, oat and barley.

The invention also relates to amino acid sequences of endoxylanase inhibitors from wheat comprising the complement of SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 19, SEQ ID No. 20, SEQ ID No. 21, SEQ ID No. 22 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions with SEQ ID No. 10, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 and SEQ ID No. 39.

The invention also relates to amino acid sequences of an endoxylanase inhibitor from barley comprising the complement of SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions with SEQ ID No. 14.

The invention also relates to amino acid sequences of endoxylanase inhibitors from rye, rice, maize and oat comprising the complement of SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 28, SEQ ID No. 30, SEQ ID No. 41, SEQ ID No. 43 or variants thereof. In addition, the invention features polynucleotide sequences which hybridize under stringent conditions with SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 27, SEQ ID No. 29, SEQ ID No. 40 and SEQ ID No. 42.

Thus the invention additionally features nucleic acid sequences encoding polypeptides, oligonucleotides, peptide nucleic acids (PNA), fragments, portions or antisense molecules thereof, and expression

vectors and host cells comprising polynucleotides that encode endoxylanase inhibitors such as TAXI I.

The invention also relates to a method for obtaining said inhibitors from a micro-organism, such as a genetically modified micro-organism which expresses said inhibitors, from a plant, or from a plant material (such as cereals, cereal grains, cereal germs or fractions thereof, cereal flours or fractions thereof, by subjecting said plant, said plant material and/or said micro-organism to one or more extraction and/or fractionation steps).

Another aspect of the present invention is related to a method for genetically transforming a micro-organism, a plant or a plant material in order to obtain the expression of the inhibitors according to the invention wherein the micro-organism, the plant or plant material is genetically modified by the introduction of a genetic material encoding said inhibitors into the micro-organism, the plant or plant material and obtain their translation and expression by genetic engineering methods well known by the man skilled in the art.

The invention furthermore relates to processes aiming at changing, preferably reducing or increasing levels of said inhibitors in a micro-organism, a plant or a plant material, by reducing or increasing the expression of said inhibitors, by the methods well known by the man skilled in the art and/or by using molecules which are able to block the inhibitor activity or activate said inhibitor.

The invention furthermore relates to use of said inhibitors for screening enzymes, such as endoxylanases that are totally, more, less or not inhibited by said inhihibitors or for modifying these enzymes, such as endoxylanases, by the methods well known by the man skilled in the art, in such way that they are totally, more, less or not inhibited by said inhibitors.

The invention furthermore relates to the obtained inhibitors, micro-organisms, plants, plant materials, and/or fractions thereof and to their use in different areas of food, feed and/or beverage technologies, such as improving malting and brewing, improving animal feedstuffs efficiency, baked and/or extruded cereal products (such as straight dough, sponge and dough and Chorleywood breads, breakfast cereals, different types of biscuits, pasta and noodles), improving the production of refrigerated and/or frozen doughs, such as to reduce syruping, (all said dough compositions comprising water and flour), improving the production of starch derived syrups, sorbitol, xylose and/or xylitol, improving wheat gluten-starch separation and production, maize processing, improving plant disease resistance, improving nutraceutical or pharmaceutical applications (such as maintaining the structure of dietary fiber material), and improving paper and pulp technologies.

The present invention will be described in details in the following description of a preferred embodiment without limiting the scope of the present invention.

Detailed description of the invention

The inventors unexpectedly found that, using an endoxylanase, such as the *Bacillus subtilis* endoxylanase, on top of the previously described *A. niger* endoxylanase for screening the inhibition activity during the isolation process of TAXI [14], the endoxylanase inhibitor (TAXI), as described by Debyser and Delcour [14] and Debyser et al. [17], is in fact a mixture of at least two endoxylanase inhibitors, i.e. TAXI I and TAXI II. Both inhibitors unexpectedly were shown to have comparable molecular masses and structures but they clearly differ from one another in pI

and endoxylanase specificity. In this way, a combination of two or more endoxylanases may be used for the isolation of endoxylanase inhibitors with a varying selectivity towards endoxylanases. It follows that the use of more endoxylanases can facilitate the identification and/or purification of inhibitors in mixtures of endoxylanase inhibitors.

The present invention for the first time shows that, on the one hand, wheat contains at least two types of TAXI-like endoxylanase inhibitors that differ in their endoxylanase specificity and that, on the other hand, at least one such inhibitor occurs in barley.

We unexpectedly found that, depending on the endoxylanase used for studying the type of inhibition, either a competitive or a non-competitive type of inhibition can be observed.

Furthermore, we describe for the first time a method for the purification of endoxylanase inhibitors, comprising of an affinity chromatographic step with immobilised endoxylanases.

We also document for the first time a new technique for the purification of endoxylanases from commercially available enzyme preparations based on affinity chromatography with an immobilised cocktail of 'TAXI'-like endoxylanase-inhibitors.

DNA sequences coding for endoxylanase inhibitors or part thereof are determined. For the first time, a recombinant active endoxylanase inhibitor from wheat was produced by a micro-organism.

DEFINITIONS

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, recombinant DNA (e.g. DNA prepared by use of recombinant DNA techniques), synthetic DNA, and RNA, as well as combinations thereof. Preferably, the term "nucleotide sequence" means DNA. The nucleotide sequences of the present invention may be single or double stranded. The nucleotide sequences of the present invention may include within them synthetic or modified nucleotides. A number of different types of modifications to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5'ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out to enhance the in vivo activity or life span of nucleotide sequences of the present invention.

The terms "variant" or "homologue" with respect to the nucleotide sequence of the present invention and the amino acid sequence of the present invention are synonymous with allelic variations of the sequences.

In particular, the term "homology" as used herein may be equated with the term "identity".

Furthermore, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of present invention, which will be limited only by the appending claims.

Examples

In what follows, the purification and partial characterisation of two endoxylanase inhibitors from wheat(*Triticum aestivum* L., var. Soissons), TAXI I and TAXI II (Example 1), and one endoxylanase inhibitor from barley (*Hordeum vulgare* L., var. Hiro), HvXI (Example 2), will be examined. In this context, an approach to isolate the inhibitors using cation exchange and gel filtration chromatography as the main techniques will be described. Furthermore, the isolation of ('TAXI'-like) endoxylanase inhibitors from a commercial wheat flour (likely a mixture of different wheat varieties), rye flour and barley whole meal using an alternative approach, i.e. affinity chromatography with immobilised endoxylanase will be discussed (Example 3). Furthermore, a new method based on affinity chromatography with immobilised 'TAXI'-like endoxylanase inhibitors to isolate endoxylanases from commercially available enzyme preparations will be examplified (example 4). We also describe corresponding DNA sequences (Examples 5,6,7,8,9 and 10) and recombinant expression of endoxylanase inhibitors (Example 11).

General experimental methods for examples 1 and 2

Materials

All reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade, unless specified otherwise.

Endoxylanase (EC: 3.2.1.8) M1 from *Trichoderma viride* (family 11), endoxylanase M4 and α-L-arabinofuranosidase (arabinofuranosidase, EC: 3.2.1.55) from *Aspergillus niger* (family 11), endoxylanase M6 from a rumen microorganism culture filtrate, azurine-crosslinked wheat AX

tablets (AZCL-AX) and soluble wheat AX (medium viscosity) were from Megazyme (Bray, Ireland). Endoxylanases from *Bacillus subtilis* (family 11) and *Aspergillus aculeatus* (family 10) were from NV Puratos (Groot-Bijgaarden, Belgium). A β-D-xylosidase (xylosidase, EC 3.2.1.37) from *A. niger* and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Bornem, Belgium). The digoxigenin (DIG) glycan detection kit® was from Boehringer (Mannheim, Germany).

All electrophoresis media and markers, chromatographic media and nitrocellulose blot membranes were from Pharmacia Biotech (Uppsala, Sweden).

Wheat (*Triticum aestivum* L., var. Soissons) and barley (*Hordeum vulgare* L., var. Hiro), were from AVEVE (Landen, Belgium) and were milled with a Bühler MLU-202 mill (Uzwil, Switzerland) and a Cyclotec 1093 sample mill (Tecator, Hogänäs, Sweden) respectively.

Protein determination

Protein concentrations were determined according to the Coomassie Brilliant Blue method of Bradford [21] with BSA as a standard.

Endoxylanase inhibition assay procedure

The inhibition activities of a set of samples were determined with the Xylazyme-AX method as described by Debyser [16]. Solutions of the *T. viride*, the *A. niger*, the *A. aculeatus* and the *B. subtilis* endoxylanases were prepared in sodium acetate buffer (25.0 mM, pH 5.0) with BSA (0.5 mg/ml) whereas the solution of the rumen microorganism culture filtrate was prepared in sodium phosphate buffer (25 mM, pH 6.0) with BSA (0.5 mg/ml). All endoxylanase solutions contained 2.0 enzyme units per 1.0 ml. One enzyme unit corresponds to an increase in extinction at 590 nm, using the xylazyme-AX method (cfr. infra), of 1.0.

Endoxylanase solution (0.5 ml) was preincubated

for 30 min at room temperature with an equal amount of sample (same buffer as enzyme solution), possibly containing inhibition activity. The mixtures were kept at 30 °C and after 10 min an AZCL-AX tablet was added. Next, they were incubated for 60 min at 30 °C. The reaction was terminated by adding 1.0% (w/v) tris-hydroxymethylaminomethane (Tris) solution (10.0 ml) and vigorous vortex stirring. After 10 min at room temperature, the tubes were shaken vigorously and the contents filtered through a Schleicher & Schuell filter (ϕ 90 mm) (Dassel, Germany). The absorbances at 590 nm (A_{590}) were measured against a control, prepared by incubating the sample with buffer instead of enzyme solution, with an Ultraspec III® UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The difference between the absorbance values of samples and another control, prepared by using buffer instead of sample, is a measure for the inhibition activity, expressed as percent reduction of endoxylanase activity.

Arabinofuranosidase and xylosidase inhibition assay procedure

The method used was based on that by Cleemput et al. [22]. *p*-Nitrophenyl arabinose and *p*-nitrophenyl xylose were used as substrates for measuring the α-L-arabinofuranosidase and β-D-xylosidase activities respectively, in the presence or absence of inhibitor. Substrate (0.05 mmol), arabinofuranosidase (50 µl), xylosidase (1.0 ml), TAXI I (234 µg), TAXI II (580 µg) and HVXI (460 µg) were separately dissolved in Mes buffer (50 mM, pH 5.5; 5 ml). Enzyme (25 µl) and endoxylanase inhibitor (25 µl) solutions were preincubated for 30 min at room temperature. Substrate (100 µl) was added and after 30 min at 30 °C the reaction was terminated by adding 1.0% (w/v) Tris solution (1.5 ml). Finally, the absorbance at 410 nm (A_{410}) was measured against a control.

Protein electrophoresis

SDS-PAGE under non-reducing and reducing conditions was performed on 20 % polyacrylamide gels with a PhastSystem® unit (Pharmacia Biotech, Uppsala, Sweden), according to the method of Laemmli [23]. β-Mercaptoethanol [5% (v/v)] was used as reducing agent. Low molecular weight (LMW) markers were α-lactalbumin (14.0 kDa); trypsin inhibitor (20.1 kDa); carbonic anhydrase (30.0 kDa); ovalbumin (43.0 kDa); albumin (67.0 kDa); phosphorylase b (94.0 kDa). The pI of the inhibitor was determined with the same instrument using polyacrylamide gels containing ampholytes (pH 3-9) and appropriate standards (Pharmacia Biotech calibration kit, pI 3.5-9.3). All gels were silver stained according to the instructions of the manufacturer (Pharmacia Biotech, Development Technique file N° 210).

Protein sequencing

TAXI I (25 μg), TAXI II (25 μg) and HVXI (25 μg) were sub-mitted to SDS-PAGE under reducing conditions (Laemmli, 1970) [23] in a SE 600 Series gel electrophoresis unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA). The slab gel (140.0 × 160.0 × 1.5 mm) consisted of a stacking gel [3.88% (w/v) T, 1.33% (w/v) C] and a running gel [17.57% (w/v) T, 0.46% (w/v) C]. Separation was achieved by using a current of 30 mA for 4 h at room temperature. The proteins were electroblotted onto a nitrocellulose membrane with the Trans-Blot® Semi-Dry Electroforetic Transfer Cell (Bio-Rad, Nazareth, Belgium), using an electric potential difference of 10 V for 1 h at room temperature, and were subjected to Edman degradation. The N-terminal amino acid sequences were determined with an Application Biosystems 477 A Protein Sequencer, connected on line with a 120 A phenylthiohydantoin-amino-acid analyser (Perkin Elmer, Lennik, Belgium).

Glycan detection

For glycan detection, the digoxigenin (DIG) glycan assay was carried out as described by Roels and Delcour [24]. TAXI I (1.0 mg/ml), TAXI II (1.0 mg/ml), HVXI (1.0 mg/ml), the positive control protein transferrin (1.0 mg/ml) and the negative control protein creatinase (1.0 mg/ml) were separated by SDS-PAGE under reducing conditions as described above, but using the sample buffer advised by the supplier of the DIG glycan detection kit®. The proteins were electroblotted onto a nitrocellulose membrane with a semi-dry PhastTransfer® unit (Pharmacia Biotech, Uppsala, Sweden), using an electric potential difference of 20 V for 30 min at 15 °C. On the blot, the vicinal diols of the glycans were converted to aldehydes with metaperiodate and labelled with the steroid hapten DIG via hydrazide. The labelled glycoconjugates were detected with a digoxigenin specific antibody conjugated to alkaline phosphatase. In the presence of the appropriate substrate, blue-purple bands appeared where the phosphatase was present. Oxidation, labelling and detection were performed according to the kit instructions (Method B).

Partial purification of wheat and barley endoxylanase inhibitors

The extraction of wheat flour and barley whole meal, the initial concentration and partial purification steps of the wheat and barley endoxylanase inhibitors were as described by Debyser and Delcour [14] and Debyser et al. [17].

Step I. Preparation of wheat flour or barley whole meal extracts

Wheat flour (10.0 kg) or barley whole meal (10.0 kg) were suspended in 0.1% (w/v) ascorbic acid (50.0 l), extracted over-night at 7 °C and centrifuged (10,000*g*; 30 min; 7 °C). To the supernatants, 2.0 g/l $CaCl_2$ was added and

the pH's were raised to 8.5 with 2.0 N NaOH to precipitate the pectins. The extracts were left overnight (7 °C) and centrifuged (10,000 *g*, 30 min, 7 °C). The pH's were adjusted to 5.0 with 2.0 M HCl.

Step II. Concentration and partial purification by cation exchange chromatography (CEC)

At pH 5.0, proteins with endoxylanase inhibiting activity from the wheat flour and barley whole meal extracts were retained by CEC on a SP Sepharose® Fast Flow column (90×90 mm). In both cases, the column was equilibrated with sodium acetate buffer (25 mM, pH 5.0; 500.0 ml) and a protein fraction was eluted with 0.5 M NaCl (1.0 l). The eluates were dialysed against deionised water (7 °C, 48 h) and lyophilised (= CEC_{wheat} material, 17.0 g and CEC_{barley} material, 10.8 g).

Example 1 Isolation and characterization of two xylanase inhibitors from wheat (TAXI I and TAXI II).

Further purification of wheat endoxylanase inhibitors

The wheat endoxylanase inhibitors, TAXI I and TAXI II, were further purified based on the method of Debyser and Delcour [14] and Debyser et al. [17]. After each purification step, the resulting fractions were assayed for endoxylanase inhibition activity with *A. niger* and *B. subtilis* endoxylanases and the purity was checked using SDS-PAGE.

Step 1. Purification by CEC

Batches of CEC_{wheat} material (4.0 g) in sodium acetate buffer (25 mM, pH 5.0; 400.0 ml) were applied on a SP Sepharose® Fast Flow column (26×300 mm), equilibrated with sodium acetate buffer (25 mM, pH 5.0; 200.0 ml). The proteins were eluted with a linear gradient of 0.0 to 0.5 M NaCl in 800.0 ml and a flow of 1.0 ml/min. Two separate fractions, one with high inhibition activity against *B. subtilis* and *A. niger* endoxylanases and one with high activity against *B. subtilis* endoxylanase but low activity against *A. niger* endoxylanase, were dialysed against deionized water (7 °C, 48 h) and lyophilised (= CEC_{wheat} I, 4.7 g, and CEC_{wheat} II, 2.9 g, respectively).

Step 2. Purification by gel permeation chromatography (GPC)

Batches of CEC_{wheat} I (20 mg) and CEC_{wheat} II (20 mg) in sodium acetate buffer (25 mM, pH 5.0; 1.0 ml) were fractionated by GPC on a Hiprep® Sephacryl® S-100 column (26×670 mm) with the same buffer (400 ml) and a flow of 0.7 ml/min. The active fractions were pooled (= GPC_{wheat} I, 590 mg in 2500 ml, and GPC_{wheat} II, 320 mg in 1630 ml, respectively).

Step 3. Purification by CEC

GPC_{wheat} I and GPC_{wheat} II were diluted three times. Batches of the diluted GPC_{wheat} I (100.0 ml) and diluted GPC_{wheat} II (100.0 ml) were fractionated by CEC on a MonoS® HR 5/5 column (5×50 mm), equilibrated with sodium acetate buffer (25 mM, pH 4.0; 5.0 ml) and sodium phosphate buffer (20 mM, pH 6.5; 5.0 ml) respectively. The bound proteins were eluted with a linear gradient of 0.0 to 0.6 M NaCl in 60.0 ml and a flow of 1.0 ml/min. The fraction, as a result of fractionation of GPC_{wheat} I and containing inhibition activity against *B. subtilis* and *A. niger* endoxylanases, and the fraction, which resulted from fractionation of GPC_{wheat} II and had activity against *B. subtilis* endoxylanase but not against *A. niger* endoxylanase, were used for further purification of TAXI I and TAXI II respectively. They were diluted three times, acidified to pH 4.0 with 1.0 N acetic acid and chromatographed again on the same MonoS® column, equilibrated with sodium acetate buffer (25 mM, pH 4.0). The same flow and salt gradient were used. We finally obtained 12.0 mg TAXI I and 9.5 mg of TAXI II.

Inhibition type determination

For TAXI I, the inhibition kinetics was studied with the *A. niger* (Megazyme, Bray, Ireland) and the *B. subtilis* (Puratos, Groot-Bijgaarden, Belgium) endoxylanase and for TAXI II only with the *B. subtilis* endoxylanase, because of its lack of inhibition activity against the *A. niger* endoxylanase, as discussed earlier. In all cases, soluble wheat arabinoxylan was used as a substrate. For each of the inhibitor/enzyme combinations the reaction rates for different substrate and inhibitor concentrations were measured. They resulted in the corresponding Dixon and Lineweaver-Burk plots.

For determining the reaction rates, a modified Somogyi reducing sugar assay was used [25]. For this method, the following reagents were prepared: reagent A (25.0 g anhydrous sodium carbonate, 25.0 g sodium potassium tartrate and 200.0 g anhydrous sodium sulphate in 1.0 l demineralised water), reagent B (30.0 g copper sulphate pentahydrate and 4 drops concentrated sulphuric acid in 200.0 ml demineralised water), reagent C (50.0 g ammonium molybdate dissolved in 900 ml demineralised water, 42.0 ml concentrated sulphuric acid and 6.0 g sodium arsenate heptahydrate dissolved separately in 50.0 ml demineralised water were mixed and the total volume was adjusted to 1.0 l), reagent D (1.0 ml of reagent B and 25.0 ml reagent A) and reagent E (one part reagent C and four parts demineralised water).

Wheat arabinoxylan (50.0, 33.3, 25.0, 20.0, 16.6, 14.2, 12.5 and 11.1 mg) was dissolved in sodium acetate buffer (100 mM, pH 5.0; 10.0 ml). The endoxylanase and endoxylanase inhibitor solutions were prepared in the same buffer containing BSA (0.5 mg/ml). The latter solutions contained 0.0 to 11.0 μg/ml TAXI I or TAXI II. The endoxylanases were diluted to such an extent that in the above described Somogyi reducing sugar assay, carried out with 0.5 % (w/w) soluble wheat AX and in absence of endoxylanase inhibitor, an increase in extinction comparable to that of the standard curve solution with the highest xylose concentration was obtained (cfr. infra).

Wheat arabinoxylan solution (0.5 ml) was mixed with 0.1 ml sodium acetate buffer (100 mM, pH 5.0) containing BSA (0.5 mg/ml) or with 0.1 ml endoxylanase inhibitor solution and incubated at 30 °C. After 10 min endoxylanase (0.1 ml), equilibrated at the same temperature, was added. The reaction was terminated 15 min later by adding reagent D (0.5 ml), after which all tubes were boiled for 20 min. The samples were cooled at room temperature and mixed with reagent E (3.0 ml) for colour development. After 15 min the

absorbance was measured at 520 nm against a reagent blank. For the latter, sodium acetate buffer (100 mM, pH 5.0) with BSA (0.5 mg/ml) was used instead of endoxylanase and endoxylanase inhibitor. To assess the reaction rates, a xylose standard curve was prepared by replacing endoxylanase and endoxylanase inhibitor with xylose solutions prepared in the same buffer (0-250.0 µg/ml).

Results

Inhibitor purification

Using the purification method described above, and *A. niger* and *B. subtilis* endoxylanases for assaying inhibition activity, TAXI I and TAXI II were purified to homogeneity from wheat flour. After initial fractionation by cation exchange chromatography (CEC) on SP Sepharose® Fast Flow columns, two protein fractions, one with high inhibition activity against *B. subtilis* and *A. niger* endoxylanases (CEC_{wheat} I) and one with high activity against *B. subtilis* endoxylanase but much lower activity against *A. niger* endoxylanase (CEC_{wheat} II), were obtained, indicating specificity of different inhibitors present. Figure 1 shows the SP Sepharose® Fast Flow chromatogram (–) of CEC_{wheat}-material, with indication of the NaCl-gradient (–) and the inhibition activities against *B. subtilis* (•) and *A. niger* (o) endoxylanases. CEC_{wheat} I and CEC_{wheat} II eluted at NaCl concentrations of 0.12 to 0.22 M and 0.23 to 0.27 M respectively. Both CEC_{wheat} I and CEC_{wheat} II contained no significant inhibition activity against *A. aculeatus* endoxylanase. The ratio of inhibition activity against *B. subtilis* endoxylanase to inhibition activity against *A. niger* endoxylanase ($IA^{B.s.}/IA^{A.n.}$) for diluted (×100) CEC_{wheat} I and CEC_{wheat} II was 1.11 and 3.21 respectively. This difference in $IA^{B.s.}/IA^{A.n.}$ indicated that we were dealing with mixtures of

two endoxylanase inhibitors, further referred to as TAXI I and TAXI II.

From CEC_{wheat} I, TAXI I was purified by gel permeation chromatography (GPC) on a Hiprep® Sephacryl® S-100 column, at which it eluted at a volume of 127.5 to 138.5 ml (GPC_{wheat} I), followed by CEC on a MonoS® column at pH 4.0, at which it eluted at NaCl concentrations of 0.27 to 0.36 M. GPC_{wheat} I contained also TAXI II but at much lower levels than TAXI I. With CEC on MonoS®, TAXI II, characterised by a much higher $IA^{B.s.}/IA^{A.n.}$ than TAXI I (cfr. infra), resulted in an additional but smaller inhibition activity peak in the chromatogram. The final purification step was performed twice at the same pH in order to increase the purity of TAXI I. Figure 2A displays the chromatogram (–) of the final separation of TAXI I on MonoS® with indication of the NaCl-gradient (-).

TAXI II was isolated from CEC_{wheat} II in a similar way, but CEC on MonoS® was performed first at pH 6.5 and secondly at pH 4.0. CEC_{wheat} II contained, in contrast to CEC_{wheat} I, much more TAXI II than TAXI I. With GPC, TAXI II eluted at the same volume as TAXI I (GPC_{wheat} II) and with CEC on MonoS® at pH 6.5 and 4.0, TAXI II eluted at NaCl concentrations of 0.08 to 0.11 M and 0.42 to 0.49 M respectively. In analogy with the above, a small additional activity peak, caused by the presence of TAXI I, was observed with CEC on MonoS® at pH 6.5. Figure 2B displays the MonoS® CEC chromatogram (–) of almost pure TAXI II, separated at pH 4.0, with indication of the NaCl-gradient (-).

Inhibitor partial molecular characterisation

Figure 3 shows the SDS-PAGE profiles of TAXI I (A) and TAXI II (B) with in lane 1 the low molecular mass markers (the size of the markers indicated on the left), in lane 2 pure inhibitor under reducing conditions and in lane 3

pure inhibitor under non-reducing conditions. The profiles (non-reducing conditions) of purified TAXI I and TAXI II show two polypeptides of ca. 40.0 kDa. Under reducing conditions, additional 30.0 and 10.0 kDa polypeptides can be seen. These findings are in agreement with those of Debyser and Delcour [14] and Debyser et al. [17]. The pI of TAXI II is at least ca. 9.3 and is therefore higher than that of TAXI I, which has a pI of ca. 8.8.

The 30.0 and 40.0 kDa polypeptides have the same N-terminal amino acid sequences, which for TAXI I and TAXI II are : SEQ ID No. 1 and SEQ ID No. 2 respectively. The N-terminal amino acid sequences of the TAXI I and TAXI II 10.0 kDa polypeptides are SEQ ID No. 4 and SEQ ID No. 5 respectively. These data confirm the molecular structure model of TAXI by Debyser and Delcour [14] and Debyser et al. [17]. Since the N-terminal sequences of the 30.0 and 40.0 kDa polypeptides are identical, the 10.0 and 30.0 kDa polypeptides, held together by one or more disulfide bonds, are probably derived from the 40.0 kDa polypeptide by proteolytic modification.

TAXI I and TAXI II are not glycosylated, as evidenced from the DIG glycan detection kit® results. Even after 15 h of colour development, no bands appeared on the blot for both inhibitors. The positive and negative control proteins, transferrin and creatinase respectively, gave the expected results.

Inhibition activities against xylanolytic enzymes

Figures 4 and 5 show the activities of different levels of TAXI I and TAXI II respectively against five different endoxylanases, i.e. *A. aculeatus* (●), *A. niger* (■), *B. subtilis* (♦), *T. viride* (×) and rumen microorganism culture filtrate endoxylanases (▲). Except in the case of the *A. niger* endoxylanase, under the specified conditions TAXI I and TAXI II have similar inhibition activity profiles, which

are depicted in Figures 4 and 5.

TAXI I has high activities against the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanases, low activity against the rumen micro-organism endoxylanases and little if any activity against the *A. aculeatus* endoxylanase. The maxima of inhibition are slightly above 90 % for the first two endoxylanases, ca. 82 % for the *B. subtilis* endoxylanase and ca. 15 % for the rumen micro-organism endoxylanases. Under the test conditions, different levels of TAXI I (ca. 0.10, ca. 0.08 and ca. 0.20 µg respectively) reduce the activities of the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanase with 50 %.

TAXI II has high activities against the *T. viride* and the *B. subtilis* endoxylanase, low activity against the rumen micro-organism endoxylanases and little if any activity against the *A. niger* and the *A. aculeatus* endoxylanase. The maxima of inhibition are slightly above 90 % for the first endoxylanase, ca. 77 % for the *B. subtilis* endoxylanase and ca. 8 % for the rumen micro-organism endoxylanases. As for TAXI I, different quantities of TAXI II (ca. 0.07 and ca. 0.28 µg respectively) reduce the activities of the *T. viride* and the *B. subtilis* endoxylanase with 50 %.

Because after boiling (15 min, pH 5.0) no inhibition activity could be found against the mentioned endoxylanases, both inhibitors are heat sensitive.

Other xylanolytic enzymes, an arabinofuranosidase and a xylosidase from *A. niger*, were not inhibited by TAXI I and TAXI II.

Inhibition type

Figure 6 shows the Dixon plots corresponding to TAXI I and *B. subtilis* endoxylanase for substrate concentrations [S] = 5.00 (♦), 3.33 (■), 2.50 (▲), 2.00 (×), 1.67 (✱) and 1.43 (●), mg/ml wheat arabinoxylan.

Figure 7 shows the Lineweaver-Burk plots corresponding to TAXI I and *B. subtilis* endoxylanase for inhibitor quantities [I] = 0.0 (■), 0.22 (♦), 0.66 (▲) and 1.10 (×) μg

Figure 8 shows the Dixon plots corresponding to TAXI II and *B. subtilis* endoxylanase for substrate concentrations [S] = 5.00 (♦), 2.50 (▲), 1.67 (✱) and 1.25 (+) mg/ml wheat arabinoxylan.

Figure 9 shows the Lineweaver-Burk plots corresponding to TAXI II and *B. subtilis* endoxylanase for inhibitor quantities [I] = 0.0 (■), 0.49 (♦), 0.74 (▲) and 0.98 (×) μg

Figure 10 shows the Dixon plots corresponding to TAXI I and *A. niger* endoxylanase for substrate concentrations [S] = 5.00 (♦), 3.33 (●), 2.50 (▲) and 2.00 (■) mg/ml wheat arabinoxylan.

Figure 11 shows the Lineweaver-Burk plots corresponding to TAXI I and *A. niger* endoxylanase for inhibitor quantities [I] = 0.0 (■), 0.22 (♦), 0.66 (▲), 1.10 (×) μg

Depending on the endoxylanase, two different types of inhibition were observed. TAXI I and TAXI II both inhibited the *B. subtilis* endoxylanase in a non-competitive manner (Figures 6, 7, 8 and 9), where as the *A. niger* endoxylanase was inhibited competitively by TAXI I (Figures 10 and 11). For non-competitive inhibition, the Dixon plot (inverse of reaction rate, 1/V, versus inhibitor concentration, [I]) corresponding to the different substrate concentrations (Figures 6 and 8) and the Lineweaver-Burk plot (inverse of reaction rate, 1/V, versus inverse of substrate concentration, 1/[S]) corresponding to different inhibitor concentrations (Figures 7 and 9) intersect on the horizontal axis in the left quadrant. In the case of competitive inhibition, however, the curves intersect in the left

quadrant (Figure 10) and on the vertical axis (Figure 11) respectively.

Discussion

Two endoxylanase inhibitors (TAXI I and TAXI II) were purified from wheat and partially characterised. Both are non-glycosylated and have similar N-terminal amino acid sequences and SDS-PAGE profiles, indicating that there may be an evolutionary relationship between them. Their pI values are respectively ca. 8.8 and ca. 9.3 or higher. Except for the *A. niger* endoxylanase, TAXI I and TAXI II have, under the specified conditions, the same inhibition activity profiles, i.e. both inhibitors inhibited the *A. aculeatus*, *B. subtilis*, *T. viride* and rumen microorganism culture filtrate endoxylanases to a similar extent (Figures 4 and 5). In the case of the *A. niger* endoxylanase, however, TAXI I resulted in a strong inhibition where as for TAXI II little if any inhibition could be observed.

Using a BLAST (version 2.0.10) search [26] in public sequence databases, the N-terminal amino acid sequences of the 40.0 and 30.0 kDa polypeptides of TAXI I were found to be 66 % identical with internal sequences of an extracellular dermal glycoprotein precursor from *Arabidopsis thaliana* (amino acids 32-46: LLLPVTKDPSTLQYT) and a glucose-6-phosphate isomerase from *Escherichia coli* (amino acids 449-460: KDPATLDYVVPF) in a 15- and 12-amino acid overlap (amino acids 3-17 and 9-20 of TAXI I) respectively. The 40.0 and 30.0 kDa polypeptides of TAXI II are 64 % identical with an internal sequence of a ribulose-1,5-biphosphate carboxylase small subunit from *Fritillaria agrestis* (amino acids 38-51: PVTQKTATGLSTLP) in a 14-amino acid overlap (amino acids 8-21 of TAXI II).

The present results show that Debyser and Delcour [14] and Debyser et al. [17], in their reports on TAXI, probably studied a mixture of TAXI I and TAXI II.

Indeed, mixtures of these two proteins with the above mentioned inhibition specificities may very well have resulted in the observation that TAXI inhibited *B. subtilis* endoxylanase more effectively than the corresponding *A. niger* enzyme. Because the authors only screened with *A. niger* endoxylanase to purify TAXI, they probably picked up CEC_{wheat} I together with some material of CEC_{wheat} II with CEC on SP Sepharose® Fast Flow (Figure 1). Nevertheless, our observations are in line with the published [17] model for the molecular structure of the TAXI type endoxylanase inhibitors, maintaining that these proteinaceous inhibitors occur in two molecular forms A and B with a molecular mass of ca. 40.0 kDa. According to the model, following reduction with β-mercaptoethanol, form B dissociates in two fragments of ca. 10.0 and ca. 30.0 kDa, whereas the molecular mass of form A is not affected by the treatment. Since the N-terminal sequences of the ca. 30.0 and ca. 40.0 kDa polypeptides were identical, the ca. 10.0 and ca. 30.0 kDa polypeptides of form B, held together by one or more disulfide bounds, are probably derived from the ca. 40.0 kDa polypeptide (form A) by proteolytic modification. We have strong indications that form A is active as endoxylanase inhibitor, but to what extent form B is active, is not clear at present. It seems reasonable to assume that the first form (A) is a precursor of the second form (B) and that the inhibitor needs to be proteolytically modified to become more or less active. A mechanism where however a non-active protein is activated by proteolytic modification has been observed for an α-amylase inhibitor from bean (*Phaseolus vulgaris* L.) seeds [27, 28].

Studies on the inhibition type of TAXI I and TAXI II unexpectedly show that the type of inhibition depends on the endoxylanase used (Figures 6 to 11). The *A. niger* endoxylanase is inhibited by TAXI I by blocking the active site, i.e. TAXI I competes with arabinoxylan (competitive inhibition) and in the case of the *B. subtilis* endoxylanase,

both TAXI I or TAXI II and arabinoxylan can bind and this independent of the binding order, i.e. TAXI I and TAXI II do not compete with arabinoxylan (non-competitive inhibition). The last type of inhibition is however in contrast to the findings of Sørensen and Poulsen [29] and Mclauchlan et al. [19] who for endoxylanase inhibitors only observed competitive inhibition. The present unexpected finding probably can be explained by the choice of endoxylanase they used to study the inhibition kinetics.

The above described results and fig 5 demonstrate, that with the latter type of inhibition inhibitor/enzyme complexes can be formed that still have some residual activity. After all, for the *B. subtilis* endoxylanase and TAXI I or TAXI II the inhibition (non-competitive) as a function of inhibitor quantity reaches a maximum at about 80% inhibition, whereas for the *A. niger* endoxylanase and TAXI I (competitive inhibition) this is at about 95%. These residual endoxylanase activities can lead to different arabinoxylan degradation patterns and products than the endoxylanase activity in absence of TAXI I and TAXI II. This in turn implies that endoxylanase inhibitors can alter the functionality of certain endoxylanases, such as the *B. subtilis* endoxylanase, and/or can have an impact on the relative affinity and/or hydrolysis specificity and/or rate versus waterextractable and/or waterunextractable arabinoxylans.

In contrast to TAXI type endoxylanase inhibitors, the more recent inhibitor described by McLauchlan et al. [19] and Hessing and Happe [20] is monomeric and glycosylated and has a molecular mass of ca. 29.0 kDa. Its N-terminal amino acid sequence is 87% identical with a sequence of rice chitinase III in a 15-amino acid overlap and shows no homology with the reported amino acid sequences of either TAXI I or TAXI II. The monomeric endoxylanase inhibitor was found to inhibit an *A. niger* endoxylanase in a competitive

manner.

In wheat, endoxylanase inhibitors may have a dual function. They possibly play an important role in regulation of plant metabolism by inhibiting endogenous endoxylanases and/or in plant defence by inhibiting exogenous endoxylanases, produced by micro-organisms and predators. In contrast to exogenous endoxylanases, endogenous endoxylanases of wheat are less well documented apart from the work by Cleemput [30] and Cleemput et al. [22, 31] who purified two endoxylanases with different substrate specificities.

Example 2. Isolation and characterization of a xylanase inhibitor from barley (HVXI)

Further purification of a barley endoxylanase inhibitor

Barley endoxylanase inhibitor, HVXI, was further purified using a method identical to that for the further purification of TAXI II (cfr. supra). After each purification step, the resulting fractions were assayed for endoxylanase inhibition activity, both with *B. subtilis* and *A. niger* endoxylanases

Step 1. Purification by CEC

CEC_{barley} was separated in a way analogous to that of CEC_{wheat}, yielding fraction CEC_{barley}' (6.84 g).

Step 2. Purification by gel permeation chromatography (GPC)

CEC_{barley}' was separated in a way analogous to that of CEC_{wheat} I or CEC_{wheat} II, yielding fraction GPC_{barley} (640 mg in 3300 ml).

Step 3. Purification by CEC

HVXI was purified to homogeneity from GPC_{barley} much as TAXI II from GPC_{wheat} II, i.e. a first separation by CEC was performed on MonoS® at pH 6.5 followed by a second separation on the same column at pH 4.0. For both separations, the same gradient and flow were used. We finally obtained 18.0 mg HVXI.

Results

Inhibitor purification

By using an identical purification procedure as described for TAXI II, HVXI was purified from barley whole meal. Figure 12 shows the SP Sepharose® Fast Flow chromatogram

(■) of CEC_{barley}-material, with indication of the NaCl-gradient (-) and the inhibition activities against *A. niger* (o) and *B. subtilis* (•) endoxylanases. With CEC on SP Sepharose® Fast Flow the fractions with inhibition activity elute at NaCl concentration of 0.15 to 0.35 M. Compared with the separation of CEC_{wheat} on SP Sepharose® Fast Flow (cfr. Supra), a somewhat similar profile was obtained when screening with the *A. niger* enzyme, apart from a right hand shoulder, possibly indicating an additional HVXI inhibitor. In the profile resulting from screening with *B. subtilis* endoxylanase the right hand shoulder was very weak, indicating again that the method as used here allows to distinguish between inhibitors of different specificity. Figure 13 displays the chromatogram (■) of the final separation of HVXI on MonoS® with indication of the NaCl-gradient (-). With CEC on MonoS® the fractions containing inhibitor elute at NaCl concentration of 0.30 to 0.35 M.

Inhibitor partial molecular characterisation

Figure 14 shows the SDS-PAGE profile of HVXI with in lane 1 the low molecular mass markers (the size of the markers indicated on the left), in lane 2 pure inhibitor under non-reducing conditions and in lane 3 pure inhibitor under reducing conditions. Under non-reducing conditions, the purified inhibitor migrated as a double protein band with a molecular mass of ca. 40.0 kDa. In the presence of β-mercaptoethanol, the SDS-PAGE gel showed three protein bands with molecular masses of ca. 40.0 kDa; ca. 30.0 kDa and ca. 10.0 kDa. The pI of the inhibitor was at least ca. 9.3 and HVXI was not glycosylated.

The ca. 40.0 kDa and ca. 30.0 kDa polypeptides both share the same N-terminal amino acid sequence SEQ ID No. 3, indicating that the ca. 30.0 kDa polypeptide is proteolytically derived from the ca. 40.0 kDa protein. The N-

terminal amino acid sequence obtained for the ca. 10.0 kDa polypeptide is SEQ ID No. 6. The N-terminal amino acid sequences of the ca. 40.0 kDa and ca. 30.0 kDa polypeptide of HVXI show a high identity (94.4% identity in a 18-amino acid overlap and 90.0% identity in a 20-amino acid overlap respectively) with those of TAXI I and TAXI II. The N-terminal amino acid sequence of the ca. 10.0 kDa polypeptide of HVXI is less similar. It has 60.0% identity with the amino acid sequence of the ca. 10.0 kDa polypeptides of TAXI I and TAXI II in 15-amino acid overlaps.

Using a BLAST (version 2.0.10) search [26], the N-terminal sequence of the ca. 40.0 kDa and ca. 30.0 kDa polypeptides (amino acids 8-18) revealed a 72.7% identity in an 11-amino acid overlap with an internal sequence PITKDAHTSIY of a hypothetical protein from *Arabidopsis thaliana* (amino acids 344-354). The sequence of the ca. 10.0 kDa polypeptide showed 60.0% identity with the sequence GALATPGYPAAPYG of "osr40g3", a rice (*Oryza sativa* L.) protein.

Inhibition activities against xylanolytic enzymes

Figure 15 shows the activities of different levels of HVXI against five different endoxylanases, i.e. *A. aculeatus* (●), *A. niger* (■), *B. subtilis* (♦), *T. viride* (×) and rumen microorganism culture filtrate endoxylanases (▲).

HVXI has high activities against the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanases (family 11), low activity against the rumen micro-organism endoxylanases and little if any activity against the *A. aculeatus* endoxylanase (family 10). The maxima of inhibition are slightly above 95 % for the first two endoxylanases, ca. 92 % for the *B. subtilis* endoxylanase and ca. 15 % for the rumen micro-organism endoxylanases. It should be noticed that in the case of HVXI, the difference between the maxima of inhibition for the *A. niger* and the *T. viride* endoxylanases

on one hand and the maximum of inhibition for the *B. subtilis* endoxylanase on the other hand, is not as pronounced as in the case of TAXI I. Under the test conditions, different levels of HVXI (ca. 0.13, ca. 0.08 and ca. 0.27 µg respectively) reduce the activities of the *A. niger*, the *T. viride* and the *B. subtilis* endoxylanase with 50 %.

Because after boiling (15 min, pH 5.0) no inhibition activity could be found against each of the mentioned endoxylanases, HVXI is heat sensitive.

Other AX hydrolysing enzymes, like an α-arabinofuranosidase and a β-xylosidase from *A. niger*, were not inhibited.

Discussion

We have purified and partially characterized HVXI, an endoxylanase inhibitor of barley. The SP Sepharose® Fast Flow profiles of CEC_{barley} however strongly suggest the presence of an additional endoxylanase inhibitor. HVXI is strongly related to TAXI I and TAXI II, since similar characteristics, N-terminal amino acid sequences and gel profiles under reducing and non-reducing conditions have been found for these wheat endoxylanase inhibitors (cfr. supra). In contrast, the inhibitor described by McLauchlan et al. [19] and Hessing and Happe [20] is a glycosylated, monomeric (single chained), basic protein with a molecular mass of 29 kDa. In addition, its N-terminal amino acid sequence showed 86% identity with the sequence of chitinase III from rice, whereas the N-termini of HVXI did not reveal such high identity with known proteins.

We believe that, much as TAXI I and TAXI II, HVXI occurs as two molecular forms, both with a molecular mass of ca. 40.0 kDa [16]. The first form exists as a single polypeptide chain. After proteolytic modification, it is transformed into the second form, which is composed of two disulfide linked subunits of ca. 30.0 kDa and ca. 10.0 kDa.

As in the case of TAXI I and TAXI II, we have strong indications that the non-proteolytically modified form is active, but to what extent the modified one is active, is still unclear. However, also here, it is reasonable to assume that the first form A is the precursor of the second form B and that, following proteolytic modification, the inhibitor becomes more or less active.

In the presence of HVXI, different endoxylanases were inhibited to a varying degree and HVXI showed a similar effect on the endoxylanase activities as TAXI I (cfr. supra). As in the case of TAXI I and TAXI II, part of the endoxylanase selectivity may be due to the fact that the enzymes tested belong to different families, i.e. family 10 or family 11. However, other factors should not be ignored, since, in the presence of the inhibitors, not all the family 11 enzymes tested reacted in the same way.

In general, proteinaceous enzyme inhibitors may be involved in plant defence mechanisms or in regulating certain metabolic activities in the plant [32, 9]. The endoxylanase inhibitor(s) in barley may have both functions. On the one hand, they may prevent the degradation of AX by phythopathogenic microorganisms, on the other hand they may regulate the AX degradation during germination. It has been shown that in extracts of germinating barley the xylan hydrolase activity appears several days later than (1-3),(1-4)-β-glucanases [33]. Although the endoxylanase genes are transcribed about 24 hours after those of the (1-3),(1-4)-β-glucanases [34], it has been suggested that the late appearance of the endoxylanase activity is due to a strong binding of these enzymes with the (aleurone) cell walls [33, 35]. However, the presence of endoxylanase inhibitors may also explain these observations.

Example 3: Isolation of endoxylanase inhibitors from commercial wheat flour, rye flour and barley whole meal using affinity chromatography with immobilised *B. subtilis* or *A. niger* endoxylanase

Experimental methods

Materials

N-hydroxysuccinimide(NHS)-activated Sepharose® 4 Fast Flow was purchased from Pharmacia Biotech (Uppsala, Sweden). Bakery enzyme preparation, Grindamyl® H 640 was from Danisco Cultor (Brabrand, Denmark). An *A. niger* endoxlanase preparation was obtained from Quest International (Naarden, the Netherlands). Rye (*Secale cereale* L., var. Halor) was from AVEVE (Landen, Belgium) and was milled with a Bühler MLU-202 mill. All other materials were as in examples 1 and 2.

Protein determination

Protein concentrations were determined in accordance with the Coomassie Brilliant Blue method of Bradford [21] with BSA as a standard.

Endoxylanase inhibition assay procedure

The inhibition activities were determined as described in the general experimental methods for examples 1 and 2.

Protein electrophoresis and sequencing

SDS-PAGE and protein sequencing were performed as described in the general experimental methods for examples 1 and 2.

Preparation of the affinity column

B. subtilis endoxylanase (XBS) was partially purified from a commercially available bakery enzyme preparation (Grindamyl® H 640) by elution on a cation exchange column (MonoS® HR 5/5) at pH 4.0 (25 mM sodium acetate) using a linear salt gradient from 0.0 to 1.0 M NaCl.

A. niger endoxylanase (XAN) was purified from a commercial Quest preparation by anion exchange chromatography (MonoQ® HR 5/5) at pH 8.0 (25 mM Tris/HCl) using a linear salt gradient from 0.0 to 1.0 M NaCl.

NHS-activated Sepharose® 4 Fast Flow (7.0 ml) was transferred to a small column of 15.0 ml, sealable at top and bottom. The matrix was washed with 1 mM HCl solution (70.0 ml). *B. subtilis* endoxylanase (50 mg) was dissolved in sodium bicarbonate buffer (0.2 M, pH 8.3; 7.0 ml) containing 0.5 M NaCl. Just before coupling, the activated matrix was washed with the same sodium bicarbonate buffer. The XBS solution (7.0 ml) was applied on top of the matrix. When ca. 4.0 ml of the enzyme solution had entered the matrix, the column was sealed and the coupling reaction was started. Coupling was performed at room temperature for 2.5 h, while the mixture was shaken. After the incubation period, the uncoupled endoxylanase was removed by washing with an ethanol amine solution (0.5 M, pH 8.3) containing 0.5 M NaCl (35.0 ml). An additional 5.0 ml of the same ethanol amine solution was added, the column was sealed and the mixture was shaken and allowed to react for 4 h at room temperature. The ethanol amine solution was replaced once during this incubation period. Finally, the matrix was washed successively with glycine solution (0.1 M, pH 3.0) containing 0.5 M NaCl (35.0 ml) and ethanol amine solution (0.5 M, pH 8.3) containing 0.5 M NaCl (35.0 ml).

The preparation of the affinity column with immobilised *A. niger* endoxylanase used an identical

procedure, but starting from a XAN solution (50 mg in 7.0 ml).

Purification method using the XBS affinity column

Step I. Extraction and concentration

The extraction of wheat flour and barley whole meal, the successive concentration and partial purification were performed as described in the general experimental methods for examples 1 and 2 resulting in CEC_{wheat} and CEC_{barley} material. From 2.5 kg of wheat flour, approximately 4.4 g of CEC_{wheat} material was obtained.

Rye flour (2.0 kg) was extracted with 20.0 l of 0.1% (w/v) ascorbic acid in water. The extract was further treated as described for wheat and barley in the general experimental methods for examples 1 and 2 yielding the CEC_{rye} material.

Step II. Purification by affinity chromatography (AFC)

Batches of the CEC_{wheat} (400 mg), CEC_{rye} (250 mg) and CEC_{barley} (250 mg) material were dissolved in sodium acetate buffer (25 mM, pH 5.0; 25 ml, 10 ml and 10 ml respectively) containing 0.2 M NaCl and applied to the affinity column with immobilised *B. subtilis* endoxylanase (equilibrated with the same buffer) at a flow rate of 0.33 ml/min. Proteins with endoxylanase inhibiting activity were eluted with 5.0 ml of a 0.25 M Tris/HCl buffer (pH 10.0) at a flow rate of 1.0 ml/min.

The eluted fractions were neutralized immediately with acetic acid (1.0M) and dialysed against sodium acetate buffer (25 mM, pH 4.0, 48 h) or were subjected to a buffer exchange (same buffer) using a PD-10 column, resulting in the AFC_{wheat} (30.5 mg protein in 187 ml), AFC_{rye} and AFC_{barley} material, respectively.

Step IIIa. Purification of wheat endoxylanase inhibitors by CEC

Three separate batches of the AFC_{wheat} solution (62 ml) was applied on a MonoS® HR 5/5 column, previously equilibrated with sodium acetate buffer (25 mM, pH 4.0). The bound proteins were eluted with a linear gradient of 0.0-0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min. This resulted in two inhibitor protein fractions, one eluting at 0.22-0.30 M NaCl (wheat inhibitor fraction I) and one eluting at 0.36-0.44 M NaCl (wheat inhibitor fraction II). The collected inhibitor fractions I (13.8 mg protein in 36.0 ml) and II (5.5 mg protein in 33.0 ml) were dialysed against sodium acetate buffer (25 mM, pH 5.0) and sodium phosphate buffer (20 mM, pH 6.5) respectively.

In a final step wheat inhibitor fractions I and II were separated on MonoS® HR 5/5 columns, equilibrated with sodium acetate buffer (25 mM, pH 5.0) and sodium phosphate buffer (20 mM, pH 6.5) respectively. In both cases, elution was with a linear gradient of 0.0-0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min.

Step IIIb. Purification of rye endoxylanase inhibitors by CEC

The AFC_{rye} material was further fractionated on a MonoS® column, equilibrated with a 25 mM sodium acetate buffer (pH 4.0). The bound proteins were eluted with a linear gradient of 0.0 to 0.6 M NaCl in 60.0 ml at a flow rate of 1.0 ml/min and collected in 0.5 ml fractions. This resulted in four separate inhibitor solutions, which were subjected to a buffer exchange [sodium acetate buffer (25 mM, pH 5.0)] (=rye inhibitor fractions I-IV).

The different rye inhibitor fractions were seperated with the MonoS® column, equilibrated with a 25 mM sodium acetate buffer (pH 5.0) and using the same elution conditions as described above. Fractions (0.5 ml) were collected and assayed for their ability to inhibit the *A. niger*, *B. subtilis* and *T. viride* endoxylanases.

Purification method using the XAN affinity column

The CEC_{wheat} material(400 mg) was dissolved in sodium acetate buffer (25 mM, pH 5.0; 25 ml) 0.2 M NaCl and applied to the affinity column with immobilised *A. niger* endoxylanase (equilibrated with the same buffer) at a flow rate of 0.33 ml/min. A protein fraction containing endoxylanase inhibiting activity was eluted with 5.0 ml of deionised water. More endoxylanase inhibiting proteins were eluted with 5.0 ml of a 0.25 M Tris/HCl buffer (pH 10.0). The flow rate during elution was 1.0 ml/min.

Results

Purification by affinity chromatography with immobilised XBS

Figure 16 shows the SDS-PAGE profiles of wheat inhibitor fractions I and II under reducing conditions (lanes 2 and 1 respectively) and non-reducing conditions (lanes 4 and 3 respectively), of the AFC_{wheat} material, the CEC_{wheat} material and the low molecular mass markers (the size of the markers indicated on the right) under non-reducing conditions (lanes 5, 6 and 7 respectively).

SDS-PAGE analysis under reducing and non-reducing conditions of the AFC_{wheat}, AFC_{rye}, AFC_{barley} and of the wheat and rye inhibitor fractions obtained after fractionation on MonoS® at pH 4.0, showed that all isolated proteins are of the same general molecular structure as described for TAXI I and TAXI II in examples 1 and 2, i.e. a form A, which consists of a single polypeptide chain of ca. 40 kDa, and a form B, which consists of two disulfide linked subunits of ca. 30 and ca. 10 kDa. Hence, the *B. subtilis* enzyme has a high selective binding affinity for the 'TAXI'-like proteins, present in wheat, rye and barley.

Purification of wheat endoxylanase inhibitors

Figure 17 shows the chromatogram (—) and the NaCl gradient (—) of the separation of AFC_{wheat} solution on a MonoS® column at pH 4.0, resulting in inhibitor fractions I and II.

The eluate (10 µl) of the affinity column contained high inhibition activity against the *A. niger* (95.2% inhibition) and the *B. subtilis* (87.3% inhibition) endoxylanases. Wheat inhibitor fraction I (10 µl) also inhibited both enzymes to a great extent (92.1% and 84.3% inhibition respectively) whereas wheat inhibitor fraction II (20 µl) inhibited the *A. niger* endoxylanase (13.4% inhibition) much less than the *B. subtilis* endoxylanase (81.4% inhibition). Fractionation of inhibitor fraction I on a MonoS® column at pH 5.0 and similar fractionation of inhibitor fraction II at pH 6.5 resulted in two and three distinct inhibitor peaks respectively.

Figure 18 shows the chromatogram (—) and the NaCl gradient (—) of the separation of the inhibitor fraction I on a MonoS® column at pH 5.0.

Figure 19 shows the chromatogram (—) and the NaCl gradient (—) of the separation of the inhibitor fraction II on a MonoS® column at pH 6.5.

The two peaks resulting from inhibitor fraction I were very close together and difficult to separate on MonoS®. At pH values above 5.0, these inhibitors barely bound on the column. They had high inhibition activities against the *A. niger* and the *B. subtilis* endoxylanases. The first two peaks resulting from inhibitor fraction II had pronounced activities against both endoxylanases whereas the third one inhibited the *B. subtilis* endoxylanase to a much higher extent

than the *A. niger* endoxylanase. The activity against the *A. niger* enzyme probably at least partially, originated from the second peak that had a small overlap with the third one.
These findings suggest that, in commercial wheat flour, up to five, or even more, inhibitors occur. Based on their elution behaviour on MonoS® and their inhibition activities against the *A. niger* and the *B. subtilis* endoxylanases, the inhibitors from inhibitor fraction I and the inhibitor(s) corresponding to the third peak obtained by MonoS® at pH 6.5 of inhibitor fraction II, may correspond to what is considered TAXI I and TAXI II respectively, as described above for var. Soissons (Example 1).

Purification of rye endoxylanase inhibitors

Figure 20 shows the chromatogram (--) and the NaCl gradient (—) of the separation of the AFC_{rye} material on a MonoS® column at pH 4.0. This chromatogram, combined with inhibition activity measurements and SDS-PAGE analysis of the collected fractions (gels not shown), suggested the presence of several 'TAXI'-like endoxylanase inhibitors in rye (SCXI or *Secale cereale* L. xylanase inhibitor). Four rye inhibitor fractions I-IV, as indicated in figure 20, were discerned.

Figure 21 shows the NaCl gradient (—) and the chromatograms of the separations of the rye inhibitor fractions I (—), II (-- --), III (- -) and IV (〰) on MonoS® at pH 5.0. After fractionation of the rye inhibi-tor fractions I-IV at least five different inhibitor peaks SCXI I-V could be distinguished, as indicated in figure 21.

When analysed with SDS-PAGE, all the inhibitors were structurally similar to TAXI and HVXI, as described in examples 1 and 2 (gel not shown). Furthermore, SCXI IV and V were electrophoretically pure, while SCXI I, II and III still contained some minor impurities. Because of the small differences in elution volume of these inhibitors and the

similar SDS-PAGE profiles, it's possible that some of these five distinct inhibitor peaks (in particular SCXI II and III) contained more than one 'TAXI'-like inhibitor.

The different rye inhibitors, in particular SCXI IV and V, reduced the activity of the endoxylanases of *A. niger*, *B. subtilis* and *T. viride* to the same extent, indicating that these inhibitors have similar specificities. In contrast, the activities of TAXI I and II from wheat against the *A. niger* endoxylanase are clearly different, the former being a strong inhibitor of this enzyme and the latter having little if any effect on its activity. Moreover, the levels of SCXI IV and V needed to reduce the activity of the endoxylanases tested by 50%, were comparable to those needed if TAXI I and HVXI are to yield the same effect.

We can conclude that rye contains a family of endoxylanase inhibitors (with at least five members) with similar structures and specificities. These characteristics corresponds well with the properties of TAXI (I) and HVXI as described in example 1 and 2. Therefore, SCXI I-V are their rye homologues.

Some preliminary work using this approach comprising affinity chromatography with immobilised *B. subtilis* endoxylanase to purify endoxylanase inhibitors from durum wheat has shown promising results with 'TAXI'-like inhibitors binding selectively to the affinity column.

Purification by affinity chromatography with immobilised XAN

Using the affinity column with immobilised *A. niger* endoxylanase and different elution conditions, we obtained two separate inhibitor fractions. The material eluted with deionised water, contained mainly proteins of about 30 kDa (SDS-PAGE), while the protein fraction eluted with the

Tris/HCl buffer, consisted of 'TAXI'-like inhibitors. Determination of the N-terminal amino acid sequence of the 30 kDa proteins yielded sequences identical to those reported by Hessing and Happe [20] and McLauchlan et al [19]. Hence, the *A. niger* enzyme has a high selective binding affinity for the 'TAXI'-like proteins as well as for the non-'TAXI' inhibitors, present in wheat. This demonstrates that the purification of endoxylanase inhibitors using affinity chromatography with an immobilised endoxylanase is not limited to the 'TAXI'-like inhibitors.

A similar approach using affinity chromatography with immobilised endoxylanase inhibitors is expected, based on the results presented above, to be a very powerful tool for the purification of various endoxylanases (example 4).

Example 4: Isolation of an *A. niger*, var. *awamori* endoxylanase from a commercial enzyme preparation using affinity chromatography with immobilised 'TAXI'-like endoxylanase inhibitors

Experimental methods

Materials

Enzyme preparation containing endoxylanase of *A. niger*, var. *awamori* was obtained from Quest international (Naarden, Netherlands). All other materials were as described in example 1, 2 and 3.

Endoxylanase activity assay procedure

The endoxylanase activities were determined with the Xylazyme-AX method. Appropriately diluted sample (1.0 ml), containing endoxylanase and prepared in sodium acetate buffer (25 mM, pH 5.0) was incubated for 60 min at 40 °C with an AZCL-AX substrate tablet. The reaction was terminated with a 1.0% (w/w) Tris solution. The remainder of the procedure was similar to that for endoxylanase inhibition activity determination.

Protein electrophoresis

SDS-PAGE was performed as described in the general experimental methods for examples 1 and 2.

Preparation of the affinity column and endoxylanase purification method

The mixture of 'TAXI'-like endoxylanase inhibitors (42.0 mg) obtained after the affinity purification step, as described in example 3, were immobilised on the same carrier using a similar procedure as for the *B. subtilis* endoxylanase, as also explained in example 3.

The *A. niger*, var. *awamori* enzyme preparation (20.0 mg) was extracted for 30 min at room temperature with sodium acetate buffer (25 mM, pH 5.0; 5.0 ml) containing NaCl (0.2 M) and the resulting suspension was centrifuged (10000 *g*, 30 min, 7 °C). The supernatant was loaded on the affinity column with the immobilised endoxylanase inhibitors, equilibrated previously with the same NaCl containing acetate buffer. The proteins retained on the column were eluted with Tris buffer (250 mM, pH 8.0; 5.0 ml) and immediately neutralised with acetic acid solution(1.0 M).

Results

Most endoxylanase activity (ca. 94%) of the enzyme preparation was retained on the affinity column, presumably by interacting with the 'TAXI'-like endoxylanase inhibitors covalently linked to the matrix. After elution most of the endoxylanase activity (ca. 84%) could be recovered. The eluate comprised mainly of the endoxylanase, which has a relative molecular mass of ca. 23 kDa. Only three additional bands with lower molecular masses and of very low intensity could be observed.

Figure 21 shows the SDS-PAGE profiles of the fraction eluted from the affinity column (lane 1), the starting material (lane 2) and low molecular mass markers (lane 3, with the size of the markers indicated on the right).

Example 5. Wheat protein encoding DNA sequences

A BLAST search (TBLASTN 2.1.2, www.ncbi.nlm.nih.gov) in the database with non-human and non-mouse EST sequences using SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 5 (this patent application) in combination with SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 and SEQ ID No. 19 as described in the patent application by Sibbesen and Sørensen [36] produced significant alignments with 5 cDNA clones of wheat:

IDENTIFIERS dbEST Id: 5493910 EST name: WWS020.H4R000101 GenBank Acc: BE420158

IDENTIFIERS dbEST Id: 5493479 EST name: WWS016.G1R000101 GenBank Acc: BE419727

IDENTIFIERS dbEST Id: 5504159 EST name: SUN002.E06R991208 GenBank Acc: BE430407

IDENTIFIERS dbEST Id: 5452003 EST name: CSB006D03F990908 GenBank Acc: BE402285

IDENTIFIERS dbEST Id: 6889613 EST name: WHE1409_B12_C23ZS GenBank Acc: BF428535

The cDNA clones were aligned with the SEQUENCHER™ programme and using SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 5 (this patent application) in combination with SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17, SEQ ID No. 18 and SEQ ID No. 19 as described in the patent application by Sibbesen and Sørensen [36] , the amino acid sequence of TAXI I was obtained within one reading frame. The present invention thus features a TAXI I variant having the amino acid sequence shown in SEQ ID No. 7:

LPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAY
PAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAXSLXHTXFVANTTDGXKPVSKVNVGVLA
ACAPSKLLASLPRGSTGVAGLA**D**SGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGPL

PWPQFTQSMPYTPLVTKGGSPAHYISAR**S**IEVGDTRVPVSEGALATGGVMLSTRLPYVLLR
RDVYRPLVDAFTKALAAQHANGAPVARAVE**P**VAPFGV**C**YDTKTLGNNLGGYAVPNVQLGLD
GGSDWTMTGKNSMVDVKXGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKR
LGFSRLPHFTGCGGL.

The present invention thus also features a TAXI I variant having the amino acid sequence shown in Seq. ID no. 8:
LPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAY
PAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAXSLXHTXFVANTTDGXKPVSKVNVGVLA
ACAPSKLLASLPRGSTGVAGLA**N**SGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGP**V**
PWPQFTQSMPYTPLVTKGGSPAHYISAR**F**IEVGDTRVPVSEGALATGGVMLSTRLPYVLLR
RDVYRPLVDAFTKALAAQHANGAPVARAVE**A**VAPFGV**L**YDTKTLGNNLGGYAVPNVQLGLD
GGSDWTMTGKNSMVDVKXGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKR
LGFSRLPHFTGCGGL.

Or the present invention features microheterogenic TAXI I variants having the amino acid sequence shown in Seq. ID no. 9:
LPVLAPVTKDPATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAY
PAPGCPAPSCGSDKHDKPCTAYPYNPVSGACAAXSLXHTXFVANTTDGXKPVSKVNVGVLA
ACAPSKLLASLPRGSTGVAGLA**X**SGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGP**X**
PWPQFTQSMPYTPLVTKGGSPAHYISAR**X**IEVGDTRVPVSEGALATGGVMLSTRLPYVLLR
RDVYRPLVDAFTKALAAQHANGAPVARAVE**X**VAPFGV**X**YDTKTLGNNLGGYAVPNVQLGLD
GGSDWTMTGKNSMVDVKXGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKR
LGFSRLPHFTGCGGL, wherein X consists of an amino acid of the group D,N,V,L,S,F,P,A and C.

Using a BLAST search (TBLASTN 2.1.2), the overall protein sequence of TAXI I shows significant homology with carrot mRNA encoding for an extracellular dermal glycoprotein (EDGP) (44%), an *Arabidopsis thaliana* putative extracellular dermal glycoprotein precursor (F15K9.16) mRNA (41%), a soybean Bg gene for a basic 7S globulin (41%) and with a *Cicer arietinum* mRNA for a putative extracellular glycoprotein (ORF1) (41%).

On the basis of the obtained nucleotide sequences of TAXI I, two other homologous cDNA clones from rice (GenBank Number: AU068900 and AU068987) were obtained from which we could determine some more upstream lying nucleotides.

From the above, the following consensus sequence of TAXI I (SEQ ID No. 10) (including ca. 95% of the coding sequence and the poly-adenylation site) was obtained:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCTCTCGTCTGGTCCACGTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCTCCCAGCTGCGGC
AGCGATAAGCACGACAAACCGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCG
CCGCMKGGAGCCTCTYCCACACGARRTTCGTGGCCAACACCACCGACGGGARYAARCCGGT
GAGCAAGGTGAACGTCGGGGTCCTGGCGGCGTGCGCGCCGAGCAAGCTCCTGGCGTCGCTG
CCCCGGGGCTCCACGGGCGTGGCCGGGCTCGCGGACTCCGGCCTGGCGCTGCCGGCGCAGG
TGGCGTCCGCGCAGAAGGTCGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCCTGG
CGTGGCCATCTTCGGCGGCGGCCCGCTCCCGTGGCCGCAATTCACGCAGTCGATGCCCTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCCGCCAGGTCCATCGAAG
TGGGGGACACCCGCGTCCCCGTATCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTGCCCTACGTCTTGCTCCGCCGCGACGTGTACCGCCCGTTGGTGGACGCGTTC
ACCAAGGCCCTGGCGGCGCAGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGCCTG
TGGCGCCGTTCGGGGTGTGCTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGC
GGTGCCCAACGTCCAGCTGGGGCTCGATGGCGGSAGTGACTGGACGATGACCGGGAAGAAC
TCGATGGTGGACGTCAAGCMRGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGG
CGGCCGGCGACGGCAGGGCGCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGT
GCTCGACTTCGACATGGAGAAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGT
TGCGGCGGCCTGTAATAATAAATCTGTTTAACGACAGGTGGATTCGTCCACTACTGCGTGT
AATAAATAAGGGAAGAAACACTTTCCATCAGTGGTTTCAT.

As a next step, based on the consensus TAXI I sequence (SEQ ID No. 10) 4 primers were designed. Primer 1 is GCCACCTCCCTCTACACAATC (SEQ ID No. 31). Primer 2 is GTAGTGGACGAATCCACCTGTC (SEQ ID No. 32). Primer 3 is CGCAATTCACGCAGTCGATG (SEQ ID No. 33). Primer 4 is

CCCAGCGTCTTCGTGTCGTAG (SEQ ID No. 34). Primers 1 and 2 are positioned at the flanks of the sequence, while primers 3 and 4 are internal primers. Primers were ordered from Genset (Paris, France).

PCR reactions were performed in 35 µl using 0.05 Units HotStarTaq DNA Polymerase (Qiagen, Hilden ,Germany), commercially supplied buffer (Qiagen), 200 µM of each dNTP, 1 µM of each primer and 50 ng total genomic DNA as template, prepared as described [37]. The reaction mixtures were subjected to incubation for 15 min at 95°C, followed by 30 cycles of 1 min at 94°C, 90 s at 57°C, 2 min at 72°C and a final incubation for 15 min at 72°C on a UNO II thermocycler (Biometra, Göttingen, Germany).

PCR products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, California, USA). The ligation mixtures, composed of 1 µl PCR 4-TOPO vector, 1 µl salt solution (supplied in the kit) and 4 µl fresh PCR product, were incubated for 5 min at 20 °C. Two µl of the ligation mixture was added to a vial of TOP10 One Shot Chemically Competent E. coli (supplied in the kit) and incubated on ice for 10 min. Subsequently, the reactions were incubated at 42°C for 30 seconds and transferred to ice. After addition of 250 µl SOC medium (supplied in the kit) and incubation in a shaker at 37°C for 1 hour, the transformations were spread on selective (ampicillin) agar plates and incubated overnight at 37°C.

DNA sequencing of the cloned PCR products was done using QIAprep Spin Miniprep (Qiagen) purified plasmids, vector specific primer and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Sequencing gels were run on a 377 ABI PRISM DNA Sequencer (Applied Biosystems).

Results

Clones representing partial xylanase inhibitor sequences were obtained from PCR-amplified genomic DNA from bread wheat (*Triticum aestivum* cultivar Soissons and cultivar Estica), durum wheat (*Triticum durum* cultivar Mexicali) and the diploid wild wheat *Aegilops tauschii*. The xylanase inhibitor type I or II was identified by alignment of the encoded amino acid sequences with peptide sequences identified from native TAXI type I and II. Besides sequences related to type I and type II, a divergent sequence (called type III) was identified. From alignments with cDNA sequences it is clear that none of the cloned fragments contained introns.

Five clones are presented. The first three sequences represent type I inhibitors. The fourth sequence represents a type II inhibitor. The last sequence represents a type III inhibitor. The sequences of five cloned PCR products are as follows:

SEQ ID No. 15 is part of a xylanase inhibitor gene termed TAXI-I.01 from *Triticum aestivum* cultivar Soissons:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCTCTCGTCTGGTCCACGTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCGCCCAGCTGCGGC
AGCAACAGGCACAACAAGCCGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCG
CCGCAGGGAGCCTCTCCCACACGAGATTCGTGGCCAACACCACCGACGGGAGCAAGCCGGT
GAGCAAGGTGAACGTCGGGGTCCTGGCGGCGTGCGCGCCGAGCAAGCTCCTGGCGTCGCTG
CCCCGGGGCTCCACGGGCGTGGCCGGGCTCGCGAACTCCGGCTTGGCGCTGCCGGCGCAGG
TGGCATCCGCGCAGAAGGTCGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCTTGG
CGTGGCCATATTTGGCGGCGGCCCGGTCCCGTGGCCGCAATTCACGCAGTCGATGCCTTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCGGCCAGGTCCATTGTAG
TGGGGGACACCCGCGTCCCCGTATCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTACCCTACGTCTTGCTCCGCCCCGACGTGTACCGCCCGTTGATGGACGCGTTT
ACCAAGGCCCTGGCGGCGCAGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGGCTG
TGGCGCCGTTCGGGGTGTGCTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGC
GGTGCCCAACGTCCAGCTGGGGCTCGATGGCGGCAGTGACTGGACGATGACCGGGAAGAAC

TCGATGGTGGACGTCAAGCAAGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGG
CGGCCGGCGACGGCAGGGCGCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGT
GCTCGACTTCGACATGGAGAAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGT
TGCGGCGGCCTGTAATAATAAATCTGTTTAACGACAGGTGGATTCGTCCACTAC.

SEQ ID No. 16 is part of a xylanase inhibitor gene termed TAXI-I.02 from *Triticum aestivum* cultivar Estica:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCTCTCGTCTGGTCCACGTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCTCCCAGCTGCGGC
AGCGATAAGCACGACAAACCGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCG
CCGCAGGGAGCCTCTCCCACACGAGATTCGTGGCCAACACCACCGACGGGAGCAAGCCGGT
GAGCAAGGTGAACGTCGGGGTCCTGGCGGCGTGCGCGCCGAGCAAGCTCCTGGCGTCGCTG
CCCCGGGGCTCCACGGGCGTGGCCGGGCTCGCGAACTCCGGCTTGGCGCTGCCGGCGCAGG
TGGCATCCGCGCAGAAGGTCGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCCTGG
CGTGGCCATATTTGGCGGCGGCCCGGTCCCGTGGCCGCAATTCACGCAGTCGATGCCTTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCGGCCAGGTCCATTGTAG
TGGGGGACACCCGCGTCCCCGTACCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTACCCTACGTCTTGCTCCGCCCCGACGTGTACCGCCCGTTGATGGACGCGTTC
ACCAAGGCCCTGGCGGCGCAGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGGCTG
TGGCGCCGTTCGGGGTGTGCTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGC
GGTGCCCAACGTCCAGCTGGGGCTCGATGGCGGCAGTGACTGGACGATGACCGGGAAGAAC
TCGATGGTGGACGTCAAGCAAGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGG
CGGCCGGCGACGGCAGGGCGCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGT
GCTCGACTTCGACATGGAGAAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGT
TGCGGCGGCCTGTAATAATAAATCTGTTTAACGACAGGTGGATTCGTCCACTAC.

SEQ ID No. 17 is part of a xylanase inhibitor gene termed TDXI-I.01 from *Triticum durum* cultivar Mexicali:

CGCAATTCACGCAGTCGATGCCTTACACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCA
CTACATCTCGGCCAGGTCCATTGTAGTGGGGGACACCCGCGTCCCCGCCGTATCGGAGGGC
GCGCTCGCCACCGGCGGCGTGATGCTCAGCACGAGGCTACCCTACGTCTTGCTCCGCCCCG
ACGTGTACCGCCCGTTGATGGACGCGTTCACCAAGGCCCTGGCGGCGCAGCATGCCAACGG
AGCGCCCGTGGCGCGCGCAGTGGAGGCTGTGGCGCCGTTCGGGGTGTGCTACGACACGAAG
ACGCTGGGCAACAACCTCGGCGGGTACGCGGTGCCCAACGTCCAGCTGGGGCTCGATGGCG

GCAGTGACTGGACGATGACCGGGAAGAACTCGATGGTGGACGTCAAGCAAGGGACGGCGTG
CGTTGCGTTCGTGGAGATGAAGGGAGTGGCGGCCGGCGACGGCAGGGCGCCGGCGGTGATC
CTCGGAGGGGCCCAGATGGAGGACTTCGTGCTCGACTTCGACATGGAGAAGAAGCGGCTCG
GGTTTAGCAGGCTGCCGCACTTTACGGGTTGCGGCGGCCTGTAATAATAAATCTGTTTAAC
GACAGGTGGATTCGTCCACTAC.

SEQ ID No. 18 is part of a xylanase inhibitor gene termed ATXI-II.01 from *Aegilops tauschii* variety Acc2220051:

GCCACCTCCCTCTACACAATCCCCTTCCACCAGGGCGCCAGCCTCGTCCTTGACATCGCCG
GCCCGCTCGTCTGGTCCACGTGCCAGCGCGGCGATCTGCCGACAGATATCCCGTGCAGTAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCGCCCAGCTGCGGC
AGCGGCAGCCACGACAAGCAATGCACGACGTACCCATCCAACCCGGTCACCGGCGCGTGCG
CCGCCGGGAGCCTCGCCCGCACGACGCTCATAGCCGACACCACCGACGGGAATAACCCGGT
GAGCCAGGTGTACGTCCGGATCCTGGCGGCGTGCGCGCCGAGAAAGCTCCTGGCGTCGCTG
CCCCGCGGCTCCATGGGCGTCGCCGGGCTAGGGGGCTCCGGCCTGGCGCTGCCGGCGCAGG
TGGCGTCCACCCAGAAGGTCGCCAACAAGTTTCTCCTCTGCCTCCCCAGCGGCGGCCCTGG
CGTGGCCATCTTCGGCGGCGGCCCGCTCCCGTGGCCGCAATTGACGCAGTCGATGCCGTAC
ACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCACTACATCTCCGTCAAGGCCATCCAAC
TGGAGGACACCCGCGTCTCCGTCTCAGAGCGCGTGCTCGCCACCGGCGGCGTGATGCTCAG
CACGAGGCTGCCCTACGCCTTGCTCCGCCACGACGTCTACCGCCCGTTGGTGGACGCGTTC
ACCAAGGCCCTGGCGGCGCAGCCTGCCAACGGAGCGCCCGTGGCGCGCGCCGTGAAGCCTG
TGGCACCGTTCGAGCTGTGCTACGACACGAAGACGCTGGG.

SEQ ID No. 40 is part of a xylanase inhibitor gene termed TAXI-III from *Triticum aestivum* cultivar Soissons:

GCCACCTCCCTCTACACAATCCCATTCCACTACGGCGCCAACATCGTGGTCGACACCGCCG
GACCGCTCGTCTGGTCCACGTGCGCACCCGACCACCTGCCGGCGGCGTTCCCGTGCAAGAG
CGCCACCTGCAGGCTCGCGAACAAGTACCACGTCCCGAGCTGCAGCGAGAGCGCGGCTGAC
AAGCTCTGCGACCACAGTCACAAGGTGTGCAGGGCCTTCCCGTACAACCCGGTCACCGGCG
CGTGCGCGGCCGGGGACCTGATCCACACCAGGTTCGTCGCCAACACCACCGACGGAAAAAA
CCCGGTGAGCCAGGTGAACGTTCGGGCCGTGGCGGCGTGCGCGCCAAGCAAACTCCTCGAG
TCGCTGCCGCAGGGCGCCTCGGGCGTGGCGGGGCTCGCGGGCTCCGACCTGGCGCTGCCGG
CGCAGGTGGCGTCCGAGCAGAAGGTCTCCAACAAGTTCCTCCTCTGCCTCCCTCGCGGCCT
CTCAAGCGACCCCGGCGTGGCCGTCTTCGGCGGCGGCCCGCTCCACTTCATGGCGCGGCCG
GAGAGGGACTACACGAAGGAGCTGGCCTACACGCCGCTCGTCGCCAAGAAGGGCAACCCCG

CGCACTACATCTCGATCAAGTCCATCGCCGTGGAGAGCGCCCGCGTGCCCGTCCCGGCGCA
GGCGCTCGCCACCGGTGGGGCGGTGCTCTGCACGAGGTCGCCCTTCACCCTGCTCCGCTCC
GACGTGTTCCTCCCGTTGGTGGACGCGTTCACCAAGGCCCTGGCGAAGCAGGGTGCGCAGG
GCGGGCCCGTGGCGAAAGCGGTGAAGCCCTACGCGCCGTTCCAGCTGTGCTACGACACGAA
GACGCTGGG.

The encoded xylanase inhibitor amino acid sequences are as follows:

SEQ ID No. 19 is part of a xylanase inhibitor type I encoded by SEQ ID No. 15:

ATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAYPAPGCPAPSCG
SNRHNKPCTAYPYNPVSGACAAGSLSHTRFVANTTDGSKPVSKVNVGVLAACAPSKLLASL
PRGSTGVAGLANSGLALPAQVASAQKVANRFLLCLPTGGLGVAIFGGGPVPWPQFTQSMPY
TPLVTKGGSPAHYISARSIVVGDTRVPVSEGALATGGVMLSTRLPYVLLRPDVYRPLMDAF
TKALAAQHANGAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNVQLGLDGGSDWTMTGKN
SMVDVKQGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFSRLPHFTG
CGGL.

SEQ ID No. 20 is part of a xylanase inhibitor type I encoded by SEQ ID No. 16:

ATSLYTIPFHDGASLVLDVAGPLVWSTCDGGQPPAEIPCSSPTCLLANAYPAPGCPAPSCG
SDKHDKPCTAYPYNPVSGACAAGSLSHTRFVANTTDGSKPVSKVNVGVLAACAPSKLLASL
PRGSTGVAGLANSGLALPAQVASAQKVANRFLLCLPTGGPGVAIFGGGPVPWPQFTQSMPY
TPLVTKGGSPAHYISARSIVVGDTRVPVPEGALATGGVMLSTRLPYVLLRPDVYRPLMDAF
TKALAAQHANGAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNVQLGLDGGSDWTMTGKN
SMVDVKQGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKRLGFSRLPHFTG
CGGL.

SEQ ID No. 21 is part of a xylanase inhibitor type I encoded by SEQ ID No. 17:

QFTQSMPYTPLVTKGGSPAHYISARSIVVGDTRVPAVSEGALATGGVMLSTRLPYVLLRPD
VYRPLMDAFTKALAAQHANGAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNVQLGLDGG
SDWTMTGKNSMVDVKQGTACVAFVEMKGVAAGDGRAPAVILGGAQMEDFVLDFDMEKKRLG
FSRLPHFTGCGGL.

SEQ ID No. 22 is part of a xylanase inhibitor type II encoded by SEQ ID No. 18:

ATSFYTIPFHQGASLVLDIAGPLVWSTCQRGDLPTDIPCSSPTCLLANAYPAPGCPAPSCG
SGSHDKQCTTYPSNPVTGACAAGSLARTTLIADTTDGNNPVSQVYVRILAACAPRKLLASL
PRGSMGVAGLGGSGLALPAQVASTQKVANKFLLCLPSGGPGVAIFGGGPLPWPQLTQSMPY
TPLVTKGGSPAHYISVKAIQLEDTRVSVSERVLATGGVMLSTRLPYALLRHDVYRPLVDAF
TKALAAQPANGAPVARAVKPVAPFELCYDTKTL.

SEQ ID No. 41 is part of a xylanase inhibitor type III encoded by SEQ ID No. 40:

ATSLYTIPFHYGANIVVDTAGPLVWSTCAPDHLPAAFPCKSATCRLANKYHVPSCSESAAD
KLCDHSHKVCRAFPYNPVTGACAAGDLIHTRFVANTTDGKNPVSQVNVRAVAACAPSKLLE
SLPQGASGVAGLAGSDLALPAQVASEQKVSNKFLLCLPRGLSSDPGVAVFGGGPLHFMARP
ERDYTKELAYTPLVAKKGNPAHYISIKSIAVESARVPVPAQALATGGAVLCTRSPFTLLRS
DVFLPLVDAFTKALAKQGAQGGPVAKAVKPYAPFQLCYDTKTL.

Example 6. Rye protein encoding DNA sequences.

PCR was also performed on genomic DNA from rye (*Secale cereale* cultivar Halo) using primers 3 (SEQ ID No. 33) and 4 (SEQ ID No. 34) according to the procedures as descibed above. Two clones are presented. The sequences of two cloned PCR products are as follows:

SEQ ID No. 23 is an internal fragment of a xylanase inhibitor gene termed SCXI-01 from *Secale cereale* cultivar Halo:

CGCAATTCACGCAGTCGATGCAGTACACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCA
CTACATCTCGCTGAAGTCCATCAAAGTGGACAACACCGGCGTCACCGTCTCGCAGAGCGCG
TTCGCCACCGGCGGCGTGATGCTGAGCACGAGGCTGCCCTACGCCCTGCTCCGCCGCGACG
TGTACCGCCCGTTGGTGGACGCGTTCACCAAGGCCCTGGCGGCGCAGCCTGCCAACGGAGC
GCCCGTGGCGCGCGCAGTGCAGCCCGTGGCGCCGTTCGGGGTGTGCTACGACACGAAGACG
CTGGG

SEQ ID No. 24 is an internal fragment of a xylanase inhibitor gene termed SCXI-02 from Secale cereale cultivar Halo:

CGCAATTCACGCAGTCGATGCAGTACACGCCGCTCGTCACCAAGGGCGGCAGCCCCGCGCA
CTACATCTCGCTCAAGTCCATCAAAGTGGACAACACCGGCGTCACCCTCTCGCAGAGCGCG
CTCGCCACCGGCGGCGTGATGCTCAGCACGAGGCTGCCCTACGCCCTGCTCCGCAGCGACG
TGTACCGCCCGTTGGTGGACGCGTTCACCAAGGCCCTGGCGGCGCAGCCTGTCAACGGAGC
GCCCGTGGCGCGCGCGGTGAAGCCCGTGGAGCCGTTCGGGGTGTGCTACGACACGAAGACG
CTGGG

The encoded xylanase inhibitor amino acid sequences are as follows:

SEQ ID No. 25 is part of a xylanase inhibitor encoded by SEQ ID No. 23:

QFTQSMQYTPLVTKGGSPAHYISLKSIKVDNTGVTVSQSAFATGGVMLSTRLPYALLRRDV
YRPLVDAFTKALAAQPANGAPVARAVQPVAPFGVCYDTKTL.

SEQ ID No. 26 is part of a xylanase inhibitor encoded by SEQ ID No. 24:

QFTQSMQYTPLVTKGGSPAHYISLKSIKVDNTGVTLSQSALATGGVMLSTRLPYALLRSDV
YRPLVDAFTKALAAQPVNGAPVARAVKPVEPFGVCYDTKTL.

Example 7. Rice protein encoding DNA sequences.

A FASTA3 search in the EMBL ESTs library using SEQ ID No. 15 (this patent application) produced significant alignment with two non-overlapping cDNA clones of rice with accession numbers D15808 and C26221. The cDNA sequence D15808 shows an overlap with the 5' part (152 nt) of SEQ ID No. 15 (this patent application). The cDNA sequence C26221 overlaps internally with SEQ ID No. 15. Based on these rice cDNA sequences, primers 5 and 6 were designed. Primer 5 is GCGGCGACCTCGCTCTACAC (SEQ ID No. 35). Primer 6 is TGTACGGGTACGCCGTGCA (SEQ ID No. 36). These primers were used to amplify a DNA fragment from rice genomic DNA using the procedure described above. A clean PCR product was directly sequenced using the individual PCR primers as sequencing primers. The sequence is as follows:

SEQ ID No. 27 is part of a xylanase inhibitor gene termed OSXI-01 from *Oryza sativa*:

GCGGCGACCTCGCTCTACACCATCCCCGTCAGGTACTACGACAACCTCGTCGTCGACCTCG
CCGGCCCGCTCGTCTGGTCGACGTGCGCCGCCGACCACCTGCCGGCGTCGCTGTCCTGCCA
GGACCCGACGTGCGTGGTCGCCAACGCGTACCGTGCTCCGACCTGCAAGGTCACCGGCGGC
GGCGGCGACTGCAGCAAGAACGTGTGCACGGCGTACCCGTACA.

SEQ ID No. 28 is part of a xylanase inhibitor protein encoded by SEQ ID No. 27:

AATSLYTIPVRYYDNLVVDLAGPLVWSTCAADHLPASLSCQDPTCVVANAYRAPTCKVTGG
GGDCSKNVCTAYPY.

Example 8. Maize protein encoding DNA sequences.

Primers 1 and 6 were also used to amplify a DNA fragment from *Zea mays* genomic DNA using identical procedures as described. A clean PCR product was directly sequenced using the individual PCR primers as sequencing primers. The sequence is as follows:

SEQ ID No. 29 is part of a xylanase inhibitor gene termed ZMXI-01 from *Zea mays*:

GCCACCTCCCTCTACACAATCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCG
GCCCGCTCGTCTGGTCCACGTGCCAGCGCGGCGATCTGCCGACAGATATCCCGTGCAGTAG
CCCCACCTGCCTCCTCGCCAACGCCTACCCCGCCCCGGGCTGCCCCGCGCCCAGCTGCGGC
AGCGACAGGCACGACAAGCCGTGCACGGCGTACCCGTACA.

SEQ ID No. 30 is part of a xylanase inhibitor protein encoded by SEQ ID No. 29:

ATSLYTIPFHDGASLVLDVAGPLVWSTCQRGDLPTDIPCSSPTCLLANAYPAPGCPAPSCG
SDRHDKPCTAYPY.

Example 9. Barley protein encoding DNA sequences.

A BLAST search (TBLASTX 2.1.2, www.ncbi.nlm.nih.gov) in the database with non-human and non-mouse EST sequences using SEQ ID No. 6 (this patent application) produced significant alignment with a cDNA clone of barley:
IDENTIFIERS dbEST Id:5811794 EST name: HVSMEh0101D07f
GenBank Acc: BE602955

The present invention thus features a part of a HVXI variant having the amino acid sequence shown in SEQ ID No. 11:
AGFAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVGKQ
GSPAHYVSGTXIKVEDTRVPVPDRALVTGGVMLNTKLAYVLLRRDVYRPVVDAFTKALAAQ
HANGA**P**AAR**A**V**D**PVAPFGLCYDAKTLGNNLGGYSVPNVVLALDGGGEWAMTGKNSMVDVKP
GX.

The present invention thus also features a part of a HVXI variant having the amino acid sequence shown in SEQ ID No. 12:
AGFAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVGKQ
GSPAHYVSGTXIKVEDTRVPVPDRALVTGGVMLNTKLAYVLLRRDVYRPVVDAFTKALAAQ
HANGA**L**AAR**G**V**N**PVAPFGLCYDAKT**N**GNNLGGYSVPNVVLALDGGGEWAMTGKNSMVDVKP
GX.

Or the present invention features a part of microheterogenic HVXI variants having the amino acid sequence shown in SEQ ID No. 13:
AGFAGSGLALPAQVASAQKVSHRFLLCLPTGGAGVAILGGGPLPWPQFTQSMAYTPLVGKQ
GSPAHYVSGTXIKVEDTRVPVPDRALVTGGVMLNTKLAYVLLRRDVYRPVVDAFTKALAAQ
HANGA**X**AAR**X**V**X**PVAPFGLCYDAKT**X**GNNLGGYSVPNVVLALDGGGEWAMTGKNSMVDVKP
GX, wherein **X** consists of an amino acid of the group P,A,D,L,G,N and C.

The obtained consensus sequence of HVXI (SEQ ID No. 14): (including ca. 48% of the coding sequence) is as follows:

GCGGGCTTCGCGGGCTCCGGCCTGGCGCTGCCGGCGCAGGTGGCGTCCGCGCAGAAGGTCT
CCCATCGGTTCCTCCTCTGCCTCCCCACGGGCGGCGCCGGCGTGGCCATCCTCGGCGGCGG
CCCGCTCCCGTGGCCGCAGTTCACGCAGTCCATGGCCTACACCCCGCTCGTCGGCAAGCAA
GGCAGCCCCGCGCACTACGTCTCGGGCACGTNCATCAAAGTCGAGGACACCCGCGTTCCCG
TTCCGGACCGCGCGCTCGTCACCGGGGGGGTGATGCTCAACACGAAGCTGGCCTACGTCTT
GCTCCGCCGCGACGTGTACCGCCCGGTGGTGGACGCGTTCACCAAGGCCCTGGCGGCGCAG
CATGCCAACGGAGCGCCCGCGGCGCGCGCCGTGGACCCCGTGGCGCCGTTCGGGCTGTGCT
ACGACGCCAAGACGCTGGGCAACAACCTCGGCGGGTACTCGGTGCCCAACGTGGTGCTGGC
GCTCGACGGCGGGGGTGAATGGGCGATGACCGGGAAGAACTCGATGGTGGACGTCAAGCCG
GGGA.

Example 10. Oat protein encoding DNA sequences

Based on IDENTIFIERS dbEST Id: 5811794 EST name: HVSMEh0101D07f GenBank Acc: BE602955, primers 9 and 10 were designed. Primer 9 is TGGCGTCCGCGCAGAAGGTC (SEQ ID No. 44). Primer 10 is GCTTGACGTCCACCATCGAG (SEQ ID No. 45). These primers were used to amplify a DNA fragment from oat genomic DNA. The resulting PCR product was cloned according to procedures described above. The sequence is as follows:

SEQ ID No. 42 is part of a xylanase inhibitor gene termed ASXI-01 from *Avena sativa*:

TGGCGTCCGCGCAGAAGGTCGCCAAGAAGTTCCTCCTCTGCCTCTCCCGCGGCGGCGTGTA
CGGAGACGGCGTGGCCATCTTCGGCGGCGGCCCGCTCCACCTCACCGCGCAGCCGGAGACA
GACTACACGCAGTCCCTTGAGTACACGCCGCTCTTCACCAAAGAAGGCAACCCGGCGTACT
ACGTCTCGGTCAAGTCCATCGCGCTGGAGAACACCCCCGTCCCCGTCTCGACCCGCACGCT
CGACGCCGGCGGTGTGGTGCTCTGCACCAGGGTGCCATACACCTTTCTCCGCCCCGACGTG
TACCTCCCGTTCGCGGACGCGTTCCGCACGGCAATGAAGGCGCAGAAGGCGCAAGAAATGA
AGGCCGTGGCGCCATTCGGGCTGTGCTACAACACGTCGACGCTGGCCAACACGCGGCTCGG
GTACCTGGTGCCGACCGTGACGCTGGCGCTGGAAGGCGGGAAGAAGTGGACGATGACGGGC
GTCCACTCGATGGTGGACGTCAAGC.

The encoded xylanase inhibitor amino acid sequence is as follows:

SEQ ID No. 43 is part of a xylanase inhibitor encoded by SEQ ID No. 42:

ASAQKVAKKFLLCLSRGGVYGDGVAIFGGGPLHLTAQPETDYTQSLEYTPLFTKEGNPAYY
VSVKSIALENTPVPVSTRTLDAGGVVLCTRVPYTFLRPDVYLPFADAFRTAMKAQKAQEMK
AVAPFGLCYNTSTLANTRLGYLVPTVTLALEGGKKWTMTGVHSMVDVK.

Example 11. Recombinant expression of xylanase inhibitors

Materials and methods

Materials, strains and media

The TOPO TA Cloning Kit for Sequencing and the pBAD/TOPO ThioFusion Expression System were obtained from Invitrogen (Carlsbad, California, USA). The pMAL Protein Fusion and purification system was purchased from New England Biolabs (Beverly, MA, USA). The pHOS31 vector was derived from the pHEN1 plasmid [38] by insertion of the phage lambda cos site and a restriction site for *I-SceI* into the *Aat*II site.

The following *E.coli* strains were used: TOP10 (genotype : F^- *mcrA* Δ(*mmr-hsd*RMS-*mcr*BC) Φ80*lacZ*Δ*M15* Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) (Invitrogen) and XL1-Blue MRF' (genotype : Δ(*mrcA*)183Δ(*mcrCB-hsdSMR-mmr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB* $lacI^q$ *ZΔM15 Tn10*(Tet^r)]) (Stratagene, La Jolla, CA, USA). *E.coli* strain TG1 (genotype : *supE hsdΔ5 thi* Δ(*lac-proAB*) [F' *traD36 proAB* $lacI^q$ *lac ZΔM15*]) was used for expression experiments.

The LB-Amp medium (1l) had the following composition : 10 g Tryptone Peptone (Difco), 5 g Selected Yeast Extract (Gibco BRL), 10 g NaCl (Acros Organics) and 100 μg/ml filter sterilised ampicillin. For agar medium, 15 g Select Agar (Gibco BRL) was added. One liter 2 x TYA contained 16 g Tryptone Peptone, 10 g Selected Yeast Extract, 5 g NaCl and 100 μg/μl filter sterilised ampicillin. In 2 x TYAG medium is 2 X TYA supplemented with 2% glucose.

Construction of the expression plasmids

The PCR 4-TOPO vector (Invitrogen) containing part of a xylanase inhibitor gene from *Triticum aestivum* cultivar Estica (example 5) was used as template for the construction of expression plasmids. Based on the sequence (SEQ ID No. 16), 2 primers were designed.
Forward primer 7 (SEQ ID No. 37) is
CCAAGATCT**CTGCCAGTTCTGGCACCTGTGACCAAAGATCCA**GCAACCTCCCTCTACAC
Reverse primer 8 (SEQ ID No. 38) is
CCTAGATCT**TTA**CAGGCCGCCGCAACCCGTAAAG. Both primers are positioned at the flanks of the sequence and contain at their 5′ end a *Bgl*II restriction site (underlined) plus 3 extra 5′ nucleotides. The forward primer (SEQ ID No. 37)contains a 3′ end corresponding to 5′ end of SEQ ID No. 16, plus a designed sequence encoding the N-terminal amino acids from mature TAXI (bold) as revealed by N-terminal sequencing of the native protein (SEQ ID No.1) and missing from the genomic sequence (SEQ ID No. 16). The reverse primer (SEQ ID No. 38) contains a 3′ end complementary to the 3′ end of the TAXI coding sequence including a stop codon (bold). Primers were ordered from Genset (Paris, France).

PCR reactions were performed in 50 µl using 0.05 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), commercially supplied buffer (Qiagen), 200µM of each dNTP, 1 µM of each primer and 200 ng of plasmid DNA. DNA amplification was carried out in an Eppendorf Mastercycler gradient (Hamburg, Germany) through an incubation step (15 min at 95°C), followed by 25 cycles of denaturation (1 min at 94°C), annealing (90 s at 57°C) and extension (2 min at 72°C). An additional extension step (20 min at 72°C) was added.

The resulting PCR product was purified using the QIAquick PCR Purification Kit (Qiagen) and directly cloned into the pBAD/TOPO ThioFusion expression vector. The ligation mixture, containing 1 µl pBAD/Thio-TOPO vector (supplied in the kit), 3 µl MilliQ water and 2 µl purified PCR product,

was
incubated during 5 min at room temperature. The mixture was then placed on ice. Three μl of the ligation mixture was added to a vial of TOP10 One Shot Chemically Competent *E.coli* (supplied in the kit) previously mixed with 2 μl of 0.5M β-mercaptoethanol, and incubated during 30 min on ice. Subsequently, the cells were incubated during 30 s at 42°C and placed immediately back on ice. After addition of 250 μl SOC medium (supplied in the kit) and incubation in a shaker at 37°C, the transformed cells were plated on a selective (ampicillin) agar plate. Colonies were grown in liquid medium. Plasmid DNA of the resulting pBAD/Thio-TAXI transformants was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Insertions were verified by digestion with *Nru*I and by DNA sequencing using vector specific primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA). Sequencing gel was run on a 377 ABI PRISM DNA Sequencer (Applied Biosystems).

In a second step, the pBAD/Thio-TAXI vector was used as DNA source for the construction of two other expression plasmids, pHOS31-TAXI and pMAL-p2X-TAXI, respectively. Therefore, the inserted TAXI gene was cut out of the pBAD/Thio-TAXI vector with *Bgl*II . The vector was simultaneously digested with *Pvu*I to prevent self ligation and unwanted insertion in the other expression plasmids. The obtained restriction fragments were subsequently ligated overnight into a *Bgl*II cut, dephosphorylated pHOS31 vector, or a *BamH*I cut, dephosphorylated pMal-p2X plasmid. The ligation mixtures were transformed to electroporation competent XL1-Blue MRF' *E.coli* cells. The transformation mixture was spread on selective (ampicillin) agar plates. Positive clones were confirmed by restriction analysis with *Nru*I/ *BamH*I in the case of the pHOS31-TAXI constructs and with *Apa*I for the pMAL-p2X-TAXI constructs. The pMAL-p2X-TAXI construct is deposited with the Belgian Coordinated

Collection of Microorganisms under access number LMBP 4268.

Recombinant expression of TAXI from pBAD/Thio-TAXI

E.coli TOP10 cells containing the pBAD/Thio-TAXI vector were analysed for the expression of recombinant thioredoxin-TAXI fusion protein. An aliquot (100 µl) of an overnight grown culture was inoculated in 5 ml LB-Amp medium. The cultures were grown to mid-exponential phase (OD_{600nm} ~0.5) at 37°C in a shaker. Expression was induced by addition of 50 µl of a 2% arabinose solution. Cells were incubated at 37°C for another 4 hours. An aliquot (1 ml) was taken for SDS-PAGE analysis. Cells were pelleted and resuspended in 75 µl MilliQ water. The remaining cells were pelleted, resuspended in 2.5 ml lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10 mM imidazole, pH8) and incubated during 30 min on ice. Following 3 freeze-thaw cycles (at -70°C and room temperature), cells were sonicated on ice during 15 s. The resulting cleared lysate was used for measuring the endoxylanase inhibition activity.

Recombinant expression of TAXI from pHOS31-TAXI and pMAL-p2x-TAXI

Vectors, pHOS31-TAXI and pMAL-p2x-TAXI, and the corresponding parental plasmids were separately transformed into *E.coli* TG1 cells. Individual colonies were picked up, inoculated into 5 ml of 2 x TYAG and grown overnight at 37°C in a shaker. Subsequently, 500 µl of the overnight grown cultures was inoculated into 50 ml fresh 2 x TYAG medium. Cell cultures were grown at 37°C until an OD_{600nm} of ~0.6. Then, the cells were pelleted and resuspended in 50 ml 2 x TYA with 1mM IPTG. After 4 hours of incubation at 30°C in a shaker, the cells were harvested. The periplasmic protein fraction was isolated by cold osmotic shock. Therefore, cells were resuspended in 5 ml 30mM Tris-HCl, 20% sucrose, pH8. Following addition of 500 µl of a 10mM EDTA solution,

the cells were shaken at room temperature during 10 min. Subsequently, the cells were pelleted at 4°C, resuspended in 5 ml icecold 5mM $MgSO_4$ and placed on ice during 10 min while shaking. The cell suspensions were centrifuged and the resulting supernatants were used for SDS-PAGE analysis and measurement of endoxylanase inhibition activity.

Protein analysis

SDS-PAGE was performed with the Mini-PROTEAN II cell system (Biorad, USA) according to the manufacturer's instructions. Gels were stained with the SimplyBlue SafeStain following the basic protocol (Invitrogen, USA).

The endoxylanase inhibition activities were determined as described in the general experimental methods for examples 1 and 2. As little as 250 µl of an endoxylanase solution was added to an equal amount of sample. In the case of the pBAD/Thio-TAXI lysate, 50 µl of 0.5M AcOH was added to obtain a pH of about 5. The periplasmic protein fractions were used as such. The reaction was terminated by adding 2% Tris solution (5 ml) in stead of 10 ml of 1% Tris.

Purification of the recombinant MBP-TAXI fusion protein

Affinity chromatography was done to purify the MBP-TAXI fusion protein according to the method described in example 3. However, *A. niger* var. awamori endoxylanase purified as discussed in example 4 in stead of *B. subtilis* endoxylanase was immobilised. An aliquot (5 ml) of the periplasmic protein fraction of the pMAL-p2X-TAXI cells together with 800 µl protease inhibitor cocktail (1 Complete tablet (Boehringer Mannheim, Germany)and 400 µl pepstatin (5mM in ethanol) in 4 ml 200mM NaAc pH5 with 1.5M NaCl) was brought onto the column. Elution of MBP-TAXI was achieved with Tris buffer (250 mM) of pH8 in stead of pH10.

Results

Construction of the pBAD/Thio-TAXI vector and expression in *E.coli*

The PCR product, containing the complete coding sequence for a mature endoxylanase inhibitor protein from wheat and 2 additional *Bgl*II restriction sites at the flanks, was cloned in frame in a pBAD/Thio-TOPO vector under the control of the p_{BAD} promoter. DNA analysis of a retained clone revealed that there were 2 silent mutations.

The TAXI encoding DNA sequence, the flanking *Bgl*II restriction sites (bold) and the stop codon of the retained clone are represented in SEQ ID No. 39:

CCA**AGATCT**TTGCCAGTTCTGGCACCTGTGACCAAAGATCCAGCAACCTCCCTCTACACAA
TCCCCTTCCACGACGGCGCCAGCCTCGTCCTCGACGTCGCCGGCCCTCTCGTCTGGTCCAC
GTGCGATGGCGGCCAGCCGCCCGCGGAGATCCCGTGCAGCAGCCCCACCTGCCTCCTCGCC
AACGCCTACCCCGCCCCGGGCTGCCCCGCTCCCAGCTGCGGCAGCGATAAGCACGACAAAC
CGTGCACGGCGTACCCGTACAACCCGGTCAGCGGCGCGTGCGCCGCAGGGAGCCTCTCCCA
CACGAGATTCGTGGCCAACACCACCGACGGGAGCAAGCCGGTGAGCAAGGTGAACGTCGGG
GTCCTGGCAGCGTGCGCGCCGAGCAAGCTCCTAGCGTCGCTGCCCCGGGGCTCCACGGGCG
TGGCCGGGCTCGCGAACTCCGGCTTGGCGCTGCCGGCGCAGGTGGCATCCGCGCAGAAGGT
CGCCAACAGGTTCCTCCTCTGCCTCCCCACCGGCGGCCCTGGCGTGGCCATATTTGGCGGC
GGCCCGGTCCCGTGGCCGCAATTCACGCAGTCGATGCCTTACACGCCGCTCGTCACCAAGG
GCGGCAGCCCCGCGCACTACATCTCGGCCAGGTCCATTGTAGTGGGGGACACCCGCGTCCC
CGTACCGGAGGGCGCGCTCGCCACCGGCGGCGTGATGCTCAGCACGAGGCTACCCTACGTC
TTGCTCCGCCCCGACGTGTACCGCCCGTTGATGGACGCGTTCACCAAGGCCCTGGCGGCGC
AGCATGCCAACGGAGCGCCCGTGGCGCGCGCAGTGGAGGCTGTGGCGCCGTTCGGGGTGTG
CTACGACACGAAGACGCTGGGCAACAACCTCGGCGGGTACGCGGTGCCCAACGTCCAGCTG
GGGCTCGATGGCGGCAGTGACTGGACGATGACCGGGAAGAACTCGATGGTGGACGTCAAGC
AAGGGACGGCGTGCGTTGCGTTCGTGGAGATGAAGGGAGTGGCGGCCGGCGACGGCAGGGC
GCCGGCGGTGATCCTCGGAGGGGCCCAGATGGAGGACTTCGTGCTCGACTTCGACATGGAG
AAGAAGCGGCTCGGGTTTAGCAGGCTGCCGCACTTTACGGGTTGCGGCGGCCTGTAA**AGAT**
CTCCG.

Figure 21 shows the nucleotide and amino acid sequences of the PCR product. The nucleotides corresponding to TAXI SEQ ID No. 16 are indicated with a line above. The primers, containing a *Bgl*II restriction site (underlined), are represented in bold. The forward primer contains nucleotide sequences complementary to the 5' end of SEQ ID No. 16 and the N-terminal amino acids from mature TAXI missing from the genomic sequence. The reverse primer contains nucleotide sequences complementary to the 3' end of SEQ ID No. 16 and a stop codon. The TAXI amino acids are in italic.

Figure 22 shows the insertion of the PCR product in the pBAD/Thio-TOPO vector. The enterokinase recognition site and 3 C-terminal amino acids of the thioredoxin protein are also indicated.

Induction of the pBAD promoter is expected to lead to the cytoplasmic expression of a thioredoxin-TAXI fusion protein of about 55.7 kDa. SDS-PAGE analysis of the total protein fraction of the cells transformed with pBAD/Thio-TAXI showed that there was a prominent protein band of about 55.7 kDa. This protein was absent in the protein fraction of the cells containing empty pBAD/Thio vector.

Figure 23 A represents the recombinant thioredoxin-TAXI fusion protein.

Figure 24 shows the SDS-PAGE profiles of the total cell extracts of the pBAD/Thio cells and the pBAD/Thio-TAXI cells (lanes 1 and 2 respectively). The low molecular mass markers are situated in lane 5. The size of the markers is indicated on the right.

Total cell lysates were used to measure the endoxylanase inhibition activity against *Bacillus subtilis* endoxylanase. No endoxylanase inhibition activity was measured in the extract containing the thioredoxin-TAXI fusion protein and the control containing thioredoxin. It appeared that the thioredoxin-TAXI producing cells contained

large inclusion bodies, as seen by phase contrast microscopy, suggesting improper folding and aggregation of the recombinant protein.

Construction of the pHOS31-TAXI and the pMAL-p2X-TAXI vectors and expression in TG1 *E.coli*

The TAXI gene (SEQ ID No. 39) was cut out the pBAD/Thio-TAXI vector with the flanking *Bgl*II restriction sites and cloned in frame into a pHOS31 and a pMAL-p2X vector.

The pHOS31 and the pMAL-p2X vector are under the control of respectively a P_{lacZ} promoter and a P_{tac} promoter and can be induced by adding IPTG. The presence of the *pelB* leader sequence and the *malE* signal sequence in respectively pHOS31 and pMAL-p2X, allow fusion proteins to be exported to the periplasm. In the case of the pHOS31-TAXI vector, a ca. 40 kDa TAXI protein will be expressed. The pMAL-p2X-TAXI construct leads to a MBP (maltose binding protein)-TAXI fusion protein of about 82 kDA. Both plasmids, pHOS31-TAXI and pMAL-p2X-TAXI, and the corresponding parental plasmids, were seperately transformed to *E.coli* TG1 cells to perform the expression experiments.

Figure 25 and 26 show the insertion of the *Bgl*II cut PCR product in respectively the *Bgl*II and the *BamH*I restriction site of respectively the pHOS31 vector and the pMAL-p2X vector. The C-terminal amino acids of the *pelB* leader sequence respectively the *malE* signal sequence together with the 'linker' amino acids are also represented.

Figure 23 B shows the recombinant TAXI protein as expressed by the pHOS31-TAXI plasmid.

Figure 23 C shows the recombinant MBP-TAXI protein as expressed by the pMAL-p2X-TAXI plasmid.

SDS-PAGE analysis of the periplasmic fractions of pHOS31-TAXI showed no clear TAXI protein band. However, the ca. 82 kDa MBP-TAXI fusion protein was prominent.

Figure 24 shows the SDS-PAGE profiles of the periplasmic protein extract with the ca. 82 kDa MBP-TAXI fusion protein or the ca. 50.8 kDa MBP control protein (lanes 4 and 3 respectively). The molecular mass markers are in lane 5 and represented on the right.

The periplasmic protein fractions of the pHOS31-TAXI and the pMAL-p2X-TAXI *E.coli* cells were used to measure the endoxylanase inhibition activity against *A.niger* and *B.subtilis* endoxylanase. Both the pHOS31-TAXI fraction and the pMAL-p2X-TAXI fraction showed endoxylanase inhibition activity against *A.niger* (96% and 99%, respectively) and *B.subtilis* (81% and 88%, respectively) endoxylanase. This indicates that a recombinant TAXI I protein was produced.

Purification of recombinant MBP-TAXI fusion protein

SDS-PAGE analysis showed that the ca. 82 kDa MBP-TAXI fusion protein was efficiently purified from the periplasmic protein fraction by affinity chromatography on immobilised *A.niger* endoxylanases. The recombinant TAXI protein has molecular form A as under reducing conditions no dissociation of the protein was noticed.

Figure 27 shows the SDS-PAGE profile of the ca. 82 kDa purified MBP-TAXI fusion protein (lane 1).The molecular mass markers are in lane 2 and indicated on the right.

Demonstration of endoxylanase inhibition activity by extracts from *E.coli* harboring the recombinant TAXI sequences was the final proof that the gene sequences identified do encode the inhibitor activity. The cloned gene can be efficiently expressed in *E.coli* into active form when secreted into the periplasm with or without a fusion protein. TAXI produced in the cytoplasm appeared inactive, most

probably due to failure of accurate disulphide bond formation and/or folding into the native state. Recombinant TAXI is active as a single chain (form A) protein.

General conclusion

As exemplified, two general methods can be used for the purification of endoxylanase inhibitors from wheat flour, and barley whole meal. Very likely, they are both also applicable for rye, durum wheat and a broad spectrum of other plant sources.

A first method comprises several (four) separations on cation exchange columns and one separation on a gel filtration column to obtain pure inhibitor. A second method comprises two purification steps, one with cation exchange chromatography and another with affinity chromatography, to obtain a pure inhibitor sample containing one or more endoxylanase inhibitors. These inhibitors can at least partially be separated from one another by further fractionations with high resolution cation exchange chromatography. This second approach to purify endoxylanase inhibitors is a highly efficient purification method since using said method we could isolate inhibitor proteins from wheat, rye and barley with large structural similarity (e.g. amino acid sequence and SDS-PAGE profile) and similarity in activity pattern as described above, as well as other endoxylanase inhibitor proteins.

In the case of wheat flour (var. Soissons) two endoxylanase inhibitors, TAXI I and TAXI II, could be purified with the first purification method and were partially characterised. Depending on the immobilised endoxylanase and the elution conditions, the second purification method allowed for the isolation of at least five inhibitors (TAXI) as well as for the isolation of other xylanase inhibitors (non-TAXI) from commercially available

wheat flour (likely a mixture of different wheat varieties).

In the case of barley whole meal (var. Hiro) one endoxylanase inhibitor, HVXI, could be purified with the first purification method and was partially characterised. However, we have indications that at least one additional endoxylanase inhibitor may be present in barley as well. Using the second method, the purification of HVXI was also successful.

In the case of rye flour (var. Halor) the second purification method resulted in several endoxylanase inhibitors (SCXI I-V), with highly similar specificities and characteristics.

The wheat, barley and rye endoxylanase inhibitors under consideration are all characterised by similar molecular masses (ca. 40.0 kDa) and structures. They occur in two different forms, i.e. proteolytically modified ones and non-modified ones. The modified forms dissociate in two polypeptides (ca. 30.0 and ca. 10.0 kDa) upon reduction with β-mercaptoethanol. Neither of the inhibitors are glycosylated. TAXI I has a pI of 8.8 where as TAXI II and HVXI have pI values of 9.3 or higher. The N-terminal amino acid sequences of these three inhibitors show a high degree of mutual identity, especially those of the ca. 40.0 kDa polypeptides, and are not described as such for proteins of other sources.

Despite the difference in pI, TAXI I and HVXI, have similar effects on the five endoxylanases mentioned above. In contrast to TAXI I and HVXI, TAXI II has only little if any inhibition activity against the *A. niger* endoxylanase, but it similarly affects the other four endoxylanases.

Studies on the inhibition type of TAXI I and TAXI II unexpectedly show that the type of inhibition depends on the endoxylanase used. The *A. niger* endoxylanase is inhibited by TAXI I by blocking the active site, i.e. TAXI I

competes with arabinoxylan, and in the case of the *B. subtilis* endoxylanase, both TAXI I or TAXI II and arabinoxylan can bind and this independent of the binding order, i.e. TAXI I and TAXI II do not compete with arabinoxylan. Because of their similar endoxylanase inhibition profiles, it is not unreasonable to assume that HVXI inhibits the *A. niger* and *B. subtilis* endoxylanases in a manner analogous to that of TAXI I.

We also document for the first time a new technique for the purification of endoxylanases from commercially available enzyme preparations based on affinity chromatography with an immobilised cocktail of 'TAXI'-like endoxylanase-inhibitors.

Furthermore, the invention features not only amino acid sequences of endoxylanase inhibitors, but also corresponding encoding polynucleotide sequences and variants, homologues or fragments thereof.

A TAXI gene was cloned and recombinant active TAXI proteins including a fusion protein were produced by *E.coli*.

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CLAIMS

1. An isolated nucleic acid molecule encoding an inhibitor, characterised in that said inhibitor inhibits cellulase, endoxylanase, β-glucanase, β-xylosidase, α-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes.
2. The isolated nucleic acid molecule of claim 1, encoding a xylanase inhibitor or a variant, homologue or fragment thereof.
3. The isolated nucleic acid molecule of claim 1 or 2, comprising a first polynucleotide sequence 70%, 80%, 90% or 95% or more identical to a second polynucleotide selected from the group consisting of: (a) a polynucleotide sequence encoding amino acids 1 to 185 of SEQ ID No. 11; (b) a polynucleotide sequence encoding amino acids 1 to 185 of SEQ ID No. 12; (c) a polynucleotide sequence encoding amino acids 1 to 185 of SEQ ID No.13; (d) a polynucleotide sequence encoding amino acids 1 to 381 of SEQ ID No. 7; (e) a polynucleotide sequence encoding amino acids 1 to 381 of SEQ ID No. 8; (f) a polynucleotide sequence encoding amino acids 1 to 381 of SEQ ID No. 9; (g) a polynucleotide sequence encoding amino acids 1 to 370 of SEQ ID No. 19; (h) a polynucleotide sequence encoding amino acids 1 to 370 of SEQ ID No. 20; (i) a polynucleotide sequence encoding amino acids 1 to 196 of SEQ ID No. 21; (j) a polynucleotide sequence encoding amino acids 1 to 277 of SEQ ID No. 22; (k) a polynucleotide sequence encoding amino acids 1 to 102 of SEQ ID No. 25; (l) a polynucleotide sequence encoding amino acids 1 to 102 of SEQ ID No. 26; (m) a polynucleotide sequence encoding amino acids 1 to 75 of SEQ ID No. 28; (n) a polynucleotide sequence encoding amino acids 1 to 74 of SEQ ID No. 30; (o) a polynucleotide sequence encoding amino acids 1 to 286 of SEQ ID No .41 and (p) a polynucleotide sequence encoding amino acids 1 to 170 of SEQ ID No. 43.
4. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (a).
5. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (b).
6. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (c).

7. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (d).
8. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (e).
9. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (f).
10. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (g).
11. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (h).
12. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (i).
13. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (j).
14. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (k).
15. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (l).
16. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (m).
17. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (n).
18. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (o).
19. The isolated nucleic acid molecule of claim 3 wherein said second polynucleotide sequence is identical to (p).
20. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (a).
21. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (b).
22. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (c).

23. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (d).
24. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (e).
25. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (f).
26. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (g).
27. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (h).
28. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (i).
29. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (j).
30. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (k).
31. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (l).
32. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (m).
33. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (n).
34. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (o).
35. The isolated nucleic acid molecule of claim 3 which comprises polynucleotide sequence (p).
36. The isolated nucleic acid molecule of claim 1 or 2 selected from the group consisting of :

(a) an isolated polynucleotide comprising a polynucleotide sequence that is at least 70%, 80%, 90% or 95% or more identical to polynucleotide sequence SEQ ID No. 10, SEQ ID No. 14., SEQ ID No. 15., SEQ ID No 16., SEQ ID No. 17., SEQ ID No. 18., SEQ ID No. 23., SEQ ID No. 24., SEQ ID No. 27., SEQ ID No 29., SEQ ID No. 39., SEQ ID

No. 40. or SEQ ID No. 42. over the entire length of SEQ ID No. 10., SEQ ID No. 14., SEQ ID No. 15., SEQ ID No 16., SEQ ID No. 17., SEQ ID No. 18., SEQ ID No. 23., SEQ ID No. 24., SEQ ID No. 27., SEQ ID No 29., SEQ ID No. 39., SEQ ID No. 40. or SEQ ID No. 42.

(b) an isolated polynucleotide which is the polynucleotide of SEQ ID No. 10., SEQ ID No. 14., SEQ ID No. 15., SEQ ID No 16., SEQ ID No. 17., SEQ ID No. 18., SEQ ID No. 23., SEQ ID No. 24., SEQ ID No. 27., SEQ ID No 29., SEQ ID No. 39., SEQ ID No. 40. or SEQ ID No. 42.

(c) an isolated polynucleotide obtainable by screening an appropriate library under stringent hybridisation conditions with a probe having the sequence of SEQ ID No. 10, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No 16, SEQ ID No. 17, SEQ ID No. 18, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 27, SEQ ID No 29, SEQ ID No. 39, SEQ ID No. 40 or SEQ ID No. 42 or a fragment thereof.

(d) An isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide that is at least 70%, 80%, 90% or 95% or more identical to the amino acid sequence over its entire length of an inhibitor of cellulolytic, xylanolytic or β-glucanolytic enzymes, with the amino acid sequence SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28. or SEQ ID No.30. ., SEQ ID No. 41. or SEQ ID No. 43.

(e) An isolated polynucleotide comprising a polynucleotide sequence encoding an inhibitor of cellulolytic, xylanolytic or β-glucanolytic enzymes, with the amino acid sequence SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30., SEQ ID No. 41 or SEQ ID No. 43.

(f) A nucleic acid sequence encoding the complete amino acid sequence encoded by the DNA contained in LMBP deposit at the Belgian Coordinated Collection of Microorganisms with deposit number LMBP 4268.

(g) An isolated polynucleotide encoding a protein expressed by the xylanase inhibitor gene

contained in plants; and

(h) A polynucleotide sequence complementary to said isolated polynucleotide of (a), (b), (c), (d), (e), (f) or (g).

37. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 10.

38. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 14.

39. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 15.

40. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 16.

41. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 17.

42. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 18.

43. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 23.

44. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 24.

45. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 27.

46. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 29.

47. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 39.

48. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 40.

49. The polynucleotide sequence of any of claims 1 to 36 capable of hybridising under stringent conditions the polynucleotide sequences SEQ ID No. 42.

50. The polynucleotide of any of the claims 1 to 49, wherein the polynucleotide is DNA.

51. The polynucleotide of any of the claims 1 to 49, wherein the polynucleotide is genomic DNA.

52. A polynucleotide probe or primer comprising at least 15 contiguous nucleotides or the polynucleotide of any of the claims 1 to 49.
53. A polynucleotide sequence according to claim 1 to 51 operably linked to a promoter.
54. A recombinant DNA construct comprising at least one of the polynucleotide sequences of claim 1 to 51.
55. The transcribed RNA product of the polynucleotide of any of the claims 1 to 51.
56. An RNA molecule or a fragment thereof which is antisense in relation to the RNA product of claim 55 and is capable of hybridising thereto.
57. A vector comprising the polynucleotide sequence according to any of the claims 1 to 51.
58. An expression system transformed with a DNA molecule according to any of the claims 1 to 51.
59. An expression system as in claim 58 and deposited with the Belgian Coordinated Collection of Microorganisms, under access number LMBP 4268.
60. A host organism transformed with the DNA molecule according to any of the claims 1 to 51.
61. The host organism of claim 60, wherein the DNA molecule according to any of the claims 1 to 51 is operably associated with a heterologous regulatory sequence.
62. The host organism of claim 60 or claim 61, wherein said host organism is a microorganism, plant, plant tissue or plant cell.
63. Method for transforming microorganisms, plants tissues or plant cells by the polynucleotides of claim 1 to claim 51, wherein the activity of the inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, α-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes is reduced.
64. Method according to claim 63, characterised in that the reduced activity of said inhibitor according to the invention is obtained by reduction of its expression.
65. Method according to claim 63, characterised in that the activity of said inhibitor is reduced by blocking the inhibitor function.
66. Method for transforming microorganisms, plants tissues or plant cells by polynucleotides of claim 1 to claim 51, wherein the activity of the inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes is increased.
67. Method according to claim 66, characterised in that increased activity of the inhibitor

according to the invention is obtained by an increase of its expression.

68. Method according to claim 66, characterised in that the activity of the inhibitor is increased by activating the inhibitor function.

69. The method of claim 66 or claim 67 for producing a recombinant protein, which is a proteinic or glycoproteinic inhibitor of cellulolytic, xylanolytic and/or β-glucanolytic enzymes, comprising: culturing a host organism comprising a nucleotide molecule according to any of the claims 1 to 51, under conditions suitable to produce said protein by said nucleic acid and recovering said protein.

70. The method of claim 69 for producing a recombinant protein, which is a proteinic or glycoproteinic inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes or fragment thereof comprising culturing the host organism of claim 60 to claim 62, whereby the recombinant protein is produced and recovering said recombinant protein.

71. The method of claim 69 or 70, comprising a method for separation or isolation of said recombinant inhibitor comprising screening the inhibition activity by using two or more enzymes during the separation or isolation steps that allow to distinguish inhibitors of different specificity.

72. The method as in claim 71 wherein the enzymes used are endoxylanases.

73. The method as in claim 72 wherein the enzymes used comprise a *Bacillus subtilis* and/or an *Aspergillus niger* endoxylanase.

74. The method as in any of the claims 69 to 73 comprising a cation-exchange chromatographic step and/or an anion-exchange chromatographic step.

75. The method as in any of the claims 69 to 71 comprising separating and/or isolating said recombinant inhibitor comprising an affinity chromatographic step with immobilised enzymes and/or antibodies against said recombinant polypeptides or fragments thereof.

76. The method as in claim 75 where the immobilised enzyme is an endoxylanase and the antibody is an antibody against said recombinant inhibitor.

77. The method as in any of the claims 75 and 76 where the immobilised endoxylanases are those of *B. subtilis* and/or *A. niger*.

78. The method as in any of the claims 75 to 77 comprising an additional cation exchange chromatographic step and/or an anion-exchange chromatographic step.

79. The method as in any of the claims 75 to 78 comprising screening the inhibition activity by using two or more enzymes during the separation and/or isolation steps that allow to distinguish inhibitors of different specificity.

80. A recombinant protein, recombinant glycoprotein or recombinant polypeptide or fragments thereof, which is an inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes or fragment thereof produced by the process of any of the claims 69 to 79.

81. A recombinant protein, recombinant glycoprotein or recombinant polypeptide or fragments thereof, which is an inhibitor of cellulase, endoxylanase, β-glucanase, β-xylosidase, β-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes or fragment thereof, coded by a nucleotide molecule according to any of the claims 1 to 51.

82. The recombinant protein of claim 80 or claim 81, which is a xylanase inhibitor.

83. The inhibitor of any of the claims 80 to 82, with an amino acid sequence 70%, 80%, 90% or 95% or more identical to SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43.

84. The inhibitor of any of the claims 80 to 83, characterised in that it has to capacity of only partially inactivating its ligand.

85. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants and/or the plant materials according to claim 69 for formation of endoxylanase-inhibitor complex wherein the inhibitor mimics the normal substrate or wherein it binds in a way that it does not prevent binding of the normal substrate.

86. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants and/or the plant materials according to claim 62 for screening endoxylanases that are totally, less or not inhibited by said inhibitor(s) or for modifying endoxylanases in such way that they are totally, less or not inhibited by said inhibitor(s).

87. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for reducing syruping

in refrigerated dough compositions, said refrigerated dough compositions comprising flour and water.

88. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for affecting the relative affinity and/or relative hydrolysis specificity and/or relative hydrolysis rate versus water-extractable and/or water-unextractable arabinoxylans of endoxylanases such as by the formation of an endoxylanase/inhibitor complex.

89. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants and/or the plant materials according to claim 62 for improving the malting of cereals such as barley, sorghum and wheat and/or the production of beer.

90. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving the production and/or quality of baked or extruded cereal products chosen among the group consisting of straight dough, sponge dough, Chorleywood bread, breakfast cereals, biscuits, pasta and noodles.

91. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant tissues according to claim 62, for improving animal feedstuff efficiency.

92. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving the production of starch derived syrups, sorbitol, xylose and/or xylitol.

93. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for wheat gluten-starch separation and production.

94. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving maize processing.

95. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving plant disease resistance.

96. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving

nutraceutical and/or pharmaceutical applications.

97. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for improving paper and pulp technologies.
98. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for purifying endoxylanases.
99. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for purifying endoxylanases in a process comprising affinity chromatography.
100. Use of the inhibitor according to any of the preceding claims 80 to 84, the micro-organisms, the plants or the plant materials according to claim 62 for purifying endoxylanases in a process comprising affinity chromatography on N-hydroxysuccinimide(NHS)-activated Sepharose® 4 Fast Flow.
101. The inhibitor as in any of the claims 80 to 84, characterised in that said inhibitor is immobilised on an affinity chromatography support.
102. A method for producing protein isolates, comprising the steps of complexing said protein with or binding said protein to said immobilised inhibitor of claim 101, and subsequent disassociation of said complex to produce the protein isolate.
103. Depletion of xylanase inhibitor ligands in a medium or mixture of compounds by complexing said xylanase inhibitor ligands with said immobilised inhibitor of claim 101, and subsequent collecting said xylanase inhibitor ligands depleted medium.
104. The method of claim 102 or claim 103, wherein said xylanase inhibitor ligands is a xylanase.
105. A preparation containing the xylanase inhibitor ligands depleted fraction obtainable by the method of claim 102 or claim 103.
106. The preparation of claim 105, wherein the xylanase inhibitor ligands depleted fraction is from a mixture of enzymes.
107. The preparation of claim 106, for modification or degradation of beta-glucan containing materials.
108. The preparation of any of the claims 105 to 107, for further isolating selected xylanases that are not inhibited by a selected xylanase inhibitor.

123. A proteinic or glycoproteinic inhibitor of cellulolytic, xylanolytic and/or β-glucanolytic enzymes, obtainable by the method of any of the claims 114 to 122, characterised by having a marker whose amino acid sequence has more than 70% homology with SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43.
124. Inhibitor as in claim 123, characterised in that the marker is the N-terminal amino acid sequence of the protein or glycoprotein.
125. Inhibitor as in claim 123 having a marker for which the amino acid sequence has more than 85% homology with SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43.
126. Inhibitor as in claim 125, characterised in that the marker is the N-terminal amino acid sequence of the protein or glycoprotein.
127. Inhibitor as in claim 123, having a marker whose amino acid sequence is identical to SEQ ID No. 1, SEQ ID No.2., SEQ ID No.3., SEQ ID No.4., SEQ ID No.5., SEQ ID No.6., SEQ ID No.7., SEQ ID No.8., SEQ ID No.9., SEQ ID No.11., SEQ ID No.12., SEQ ID No.13., SEQ ID No.19., SEQ ID No.20., SEQ ID No.21., SEQ ID No.22, SEQ ID No.25., SEQ ID No.26., SEQ ID No.28., SEQ ID No.30, SEQ ID No. 41 or SEQ ID No. 43., or a variant, homologue or fragment thereof.
128. Inhibitor as in claim 127, characterised in that the marker is the N-terminal amino acid sequence of the protein or glycoprotein.
129. Inhibitor as in any of the claims 123 to 128, characterised in that said inhibitor inhibits cellulase, endoxylanase, β-glucanase, β-xylosidase, α-L-arabino-furanosidase and/or other cellulose, xylan, arabinoxylan or β-glucan degrading enzymes.
130. Inhibitor as in any of the claims 123 to 129 or fractions thereof, characterised in that it is obtainable from plant material.

109. Preparations of any of the claims 105 to 107 characterised in that they contain xylanases that are not inhibited by selected xylanase inhibitors for degradation, modification or degradation of arabinoxylans in the presence said selected xylanase inhibitors.
110. An isolated antibody or fragment thereof, which specifically binds to said inhibitor of claim 80 to claim 84.
111. A compound, which modulates said inhibitor of any of the claims 80 to 84.
112. A compound according to claim 111 which antagonises or selectively antagonises said inhibitor of claim 80 to claim 84.
113. A compound according to claim 111, which agonises said inhibitor of claim 80 to claim 84.
114. A method for separation and/or isolation of inhibitors of cellulolytic, xylanolytic and/or β-glucanolytic enzymes comprising screening the inhibition activity by using two or more enzymes during the separation and/or isolation steps that allow to distinguish inhibitors of different specificity.
115. A method as in claim 114, wherein the enzymes used are endoxylanases.
116. A method as in claim 114 or claim 115, wherein the enzymes used comprise a *Bacillus subtilis* and/or an *Aspergillus niger* endoxylanase.
117. A method as in any of the claims 115 to 116, comprising a cation-exchange chromatographic step and/or an anion-exchange chromatographic step.
118. A method for separation and/or isolation of cellulolytic, xylanolytic and/or β-glucanolytic enzymes comprising an affinity chromatographic step with immobilised enzymes and/or antibodies against inhibitors.
119. A method as in claim 118 where the immobilised enzyme is an endoxylanase and the antibody is an antibody against the endoxylanase inhibitor.
120. A method as in any of the claims 118 and 119 where the immobilised endoxylanases are those of *B. subtilis* and/or *A. niger*.
121. A method as in any of the claims 118 to 120 comprising an additional cation-exchange chromatographic step and/or an anion-exchange chromatographic step.
122. A method as in any of the claims 118 to 121 comprising screening the inhibition activity by using two or more enzymes during the separation and/or isolation steps that allow to distinguish inhibitors of different specificity.

131. Inhibitor as in claim 129, characterised in that said plant material is selected from the group consisting of cereals, cereal grains, cereal germs or cereal flours from wheat, durum wheat, rye, triticale, barley, sorghum, oats, maize or rice.
132. Inhibitor as in any of the claims 123 to 130, characterised in that it is obtainable from microorganisms or fractions thereof.
133. Inhibitor as in any of the claims 123 to 132, characterised in that it is an endoxylanase inhibitor.
134. Inhibitor as in claim 133, characterised in that it is a water-soluble species.
135. Inhibitor as in any of the claims 123 to 134, characterised in that said protein or glycoprotein is selected from the group comprising proteins or glycoproteins having a molecular weight typically between 40 kDa and 43 kDa, proteins or glycoproteins having a molecular weight of typically 30 kDa and proteins or glycoproteins having a molecular weight of typically 10 kDa.
136. Inhibitor as in any of the claims 123 to 135, characterised in that said protein or glycoprotein typically has a molecular weight between 40 kDa and 43 kDa and a pI greater than 7 or about 7.
137. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122, for formation of endoxylanase-inhibitor complex wherein the inhibitor mimics the normal substrate or wherein it binds in a way that it does not prevent binding of the normal substrate.
138. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122, for screening endoxylanases that are totally, less or not inhibited by said inhibitor(s) or for modifying endoxylanases in such way that they are totally, less or not inhibited by said inhibitor(s).
139. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for reducing syruping in refrigerated dough compositions, said refrigerated dough compositions comprising flour and water.
140. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for affecting the relative affinity and/or relative hydrolysis specificity and/or relative hydrolysis rate versus water-extractable and/or water-unextractable arabinoxylans of endoxylanases such as by the formation of an endoxylanase/inhibitor complex.

141. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving the malting of cereals such as barley, sorghum and wheat and/or the production of beer.
142. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving the production and/or quality of baked or extruded cereal products chosen among the group consisting of straight dough, sponge dough, Chorleywood bread, breakfast cereals, biscuits, pasta and noodles.
143. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving animal feedstuff efficiency.
144. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving the production of starch derived syrups, sorbitol, xylose and/or xylitol.
145. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for wheat gluten-starch separation and production.
146. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving maize processing.
147. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving plant disease resistance.
148. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving nutraceutical and/or pharmaceutical applications.
149. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for improving paper and pulp technologies.
150. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for purifying endoxylanases.
151. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for purifying endoxylanases in a process comprising affinity chromatography.
152. Use of the inhibitor of any of the preceding claims 123 to 136 or obtained by the method of any of the claims 114 to 122 for purifying endoxylanases in a process

comprising affinity chromatography on N-hydroxysuccinimide(NHS)-activated Sepharose® 4 Fast Flow.

153. The inhibitor as in any of the claims 123 to 136, characterised in that said inhibitor is immobilised on an affinity chromatography support.
154. A method of producing protein isolates, comprising the steps of complexing said protein with or binding said protein to said immobilised inhibitor of claim 153, and subsequent disassociation of said complex to produce the protein isolate.
155. Depletion of xylanase inhibitor ligands in a medium or mixture of compounds by complexing said xylanase inhibitor ligands with said immobilised inhibitor of claim 153, and subsequent collecting said xylanase inhibitor ligands depleted medium.
156. The method of claim 154 or 155, wherein said xylanase inhibitor ligands is a xylanase.
157. A preparation containing the xylanase inhibitor ligands depleted fraction obtainable by the method of claim 155 or claim 156.
158. The preparation of claim 157, wherein the xylanase inhibitor ligands depleted fraction is from a mixture of enzymes.
159. The preparation of claim 158, for modification or degradation of beta-glucan containing materials.
160. The preparation of claim 157 to 159, for further isolating selected xylanases that are not inhibited by a selected xylanase inhibitor.
161. Preparations of claim 157 to 159 characterised in that they contain xylanases that are not inhibited by selected xylanase inhibitors for degradation, modification or degradation of arabinoxylans in the presence of said selected xylanase inhibitors.

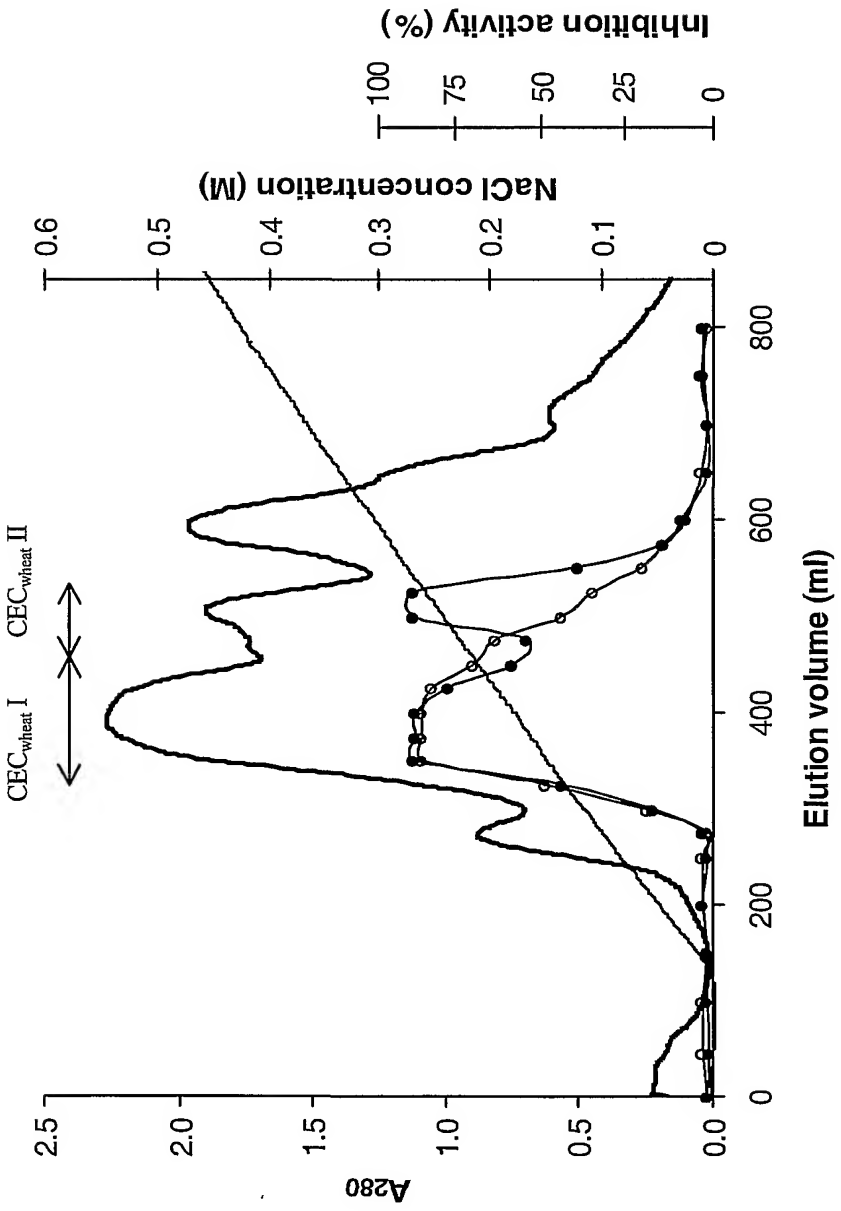
Inhibition activity (%)
100
75
50
25
0
NaCl concentration (M)
0.6
0.5
0.4
0.3
0.2
0.1
0
A280
2.5
2.0
1.5
1.0
0.5
0.0
0
200
400
600
800
Elution volume (ml)
CEC wheat I
CEC wheat II

Figure 1

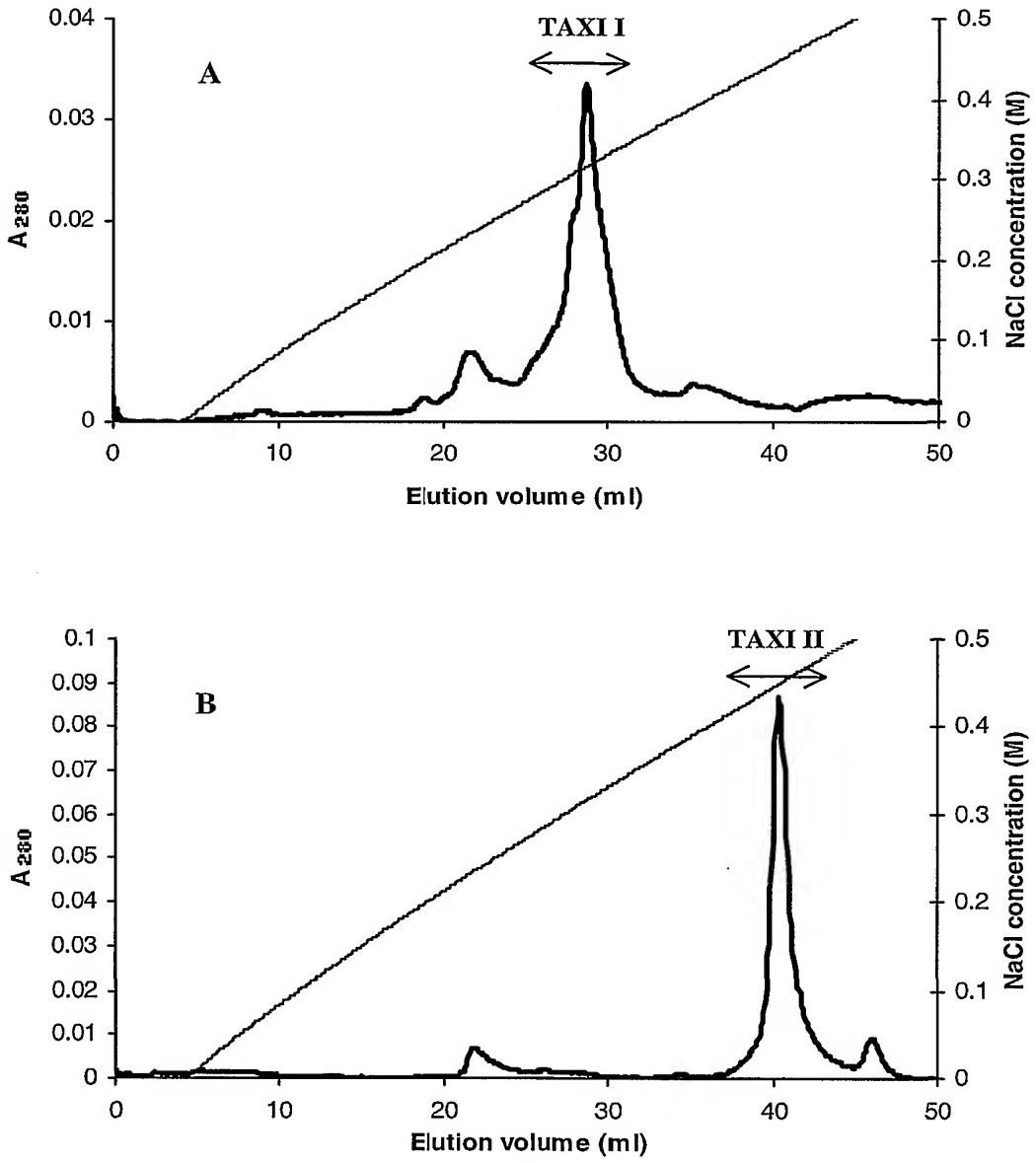
A
TAXI I
A280
NaCl concentration (M)
Elution volume (ml)
0.04
0.03
0.02
0.01
0
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
B
TAXI II
A280
NaCl concentration (M)
Elution volume (ml)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50

Figure 2

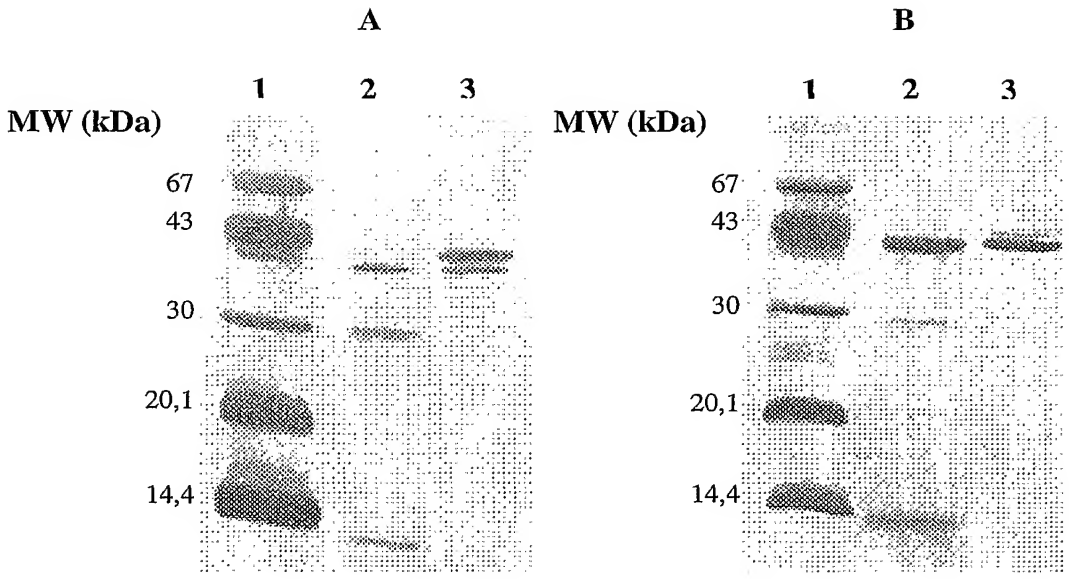

Figure 3

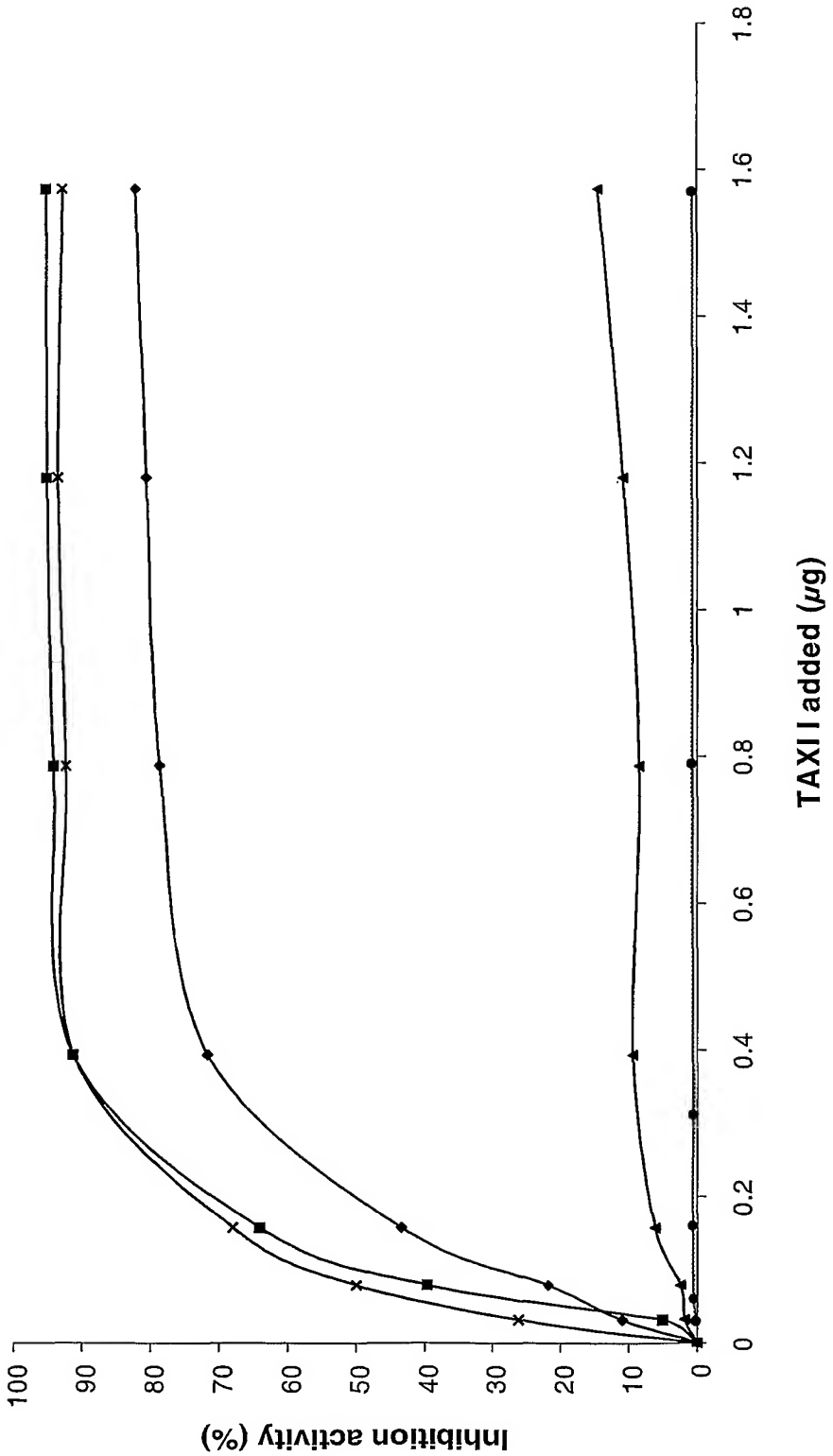
Inhibition activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
TAXI I added (µg)

Figure 4

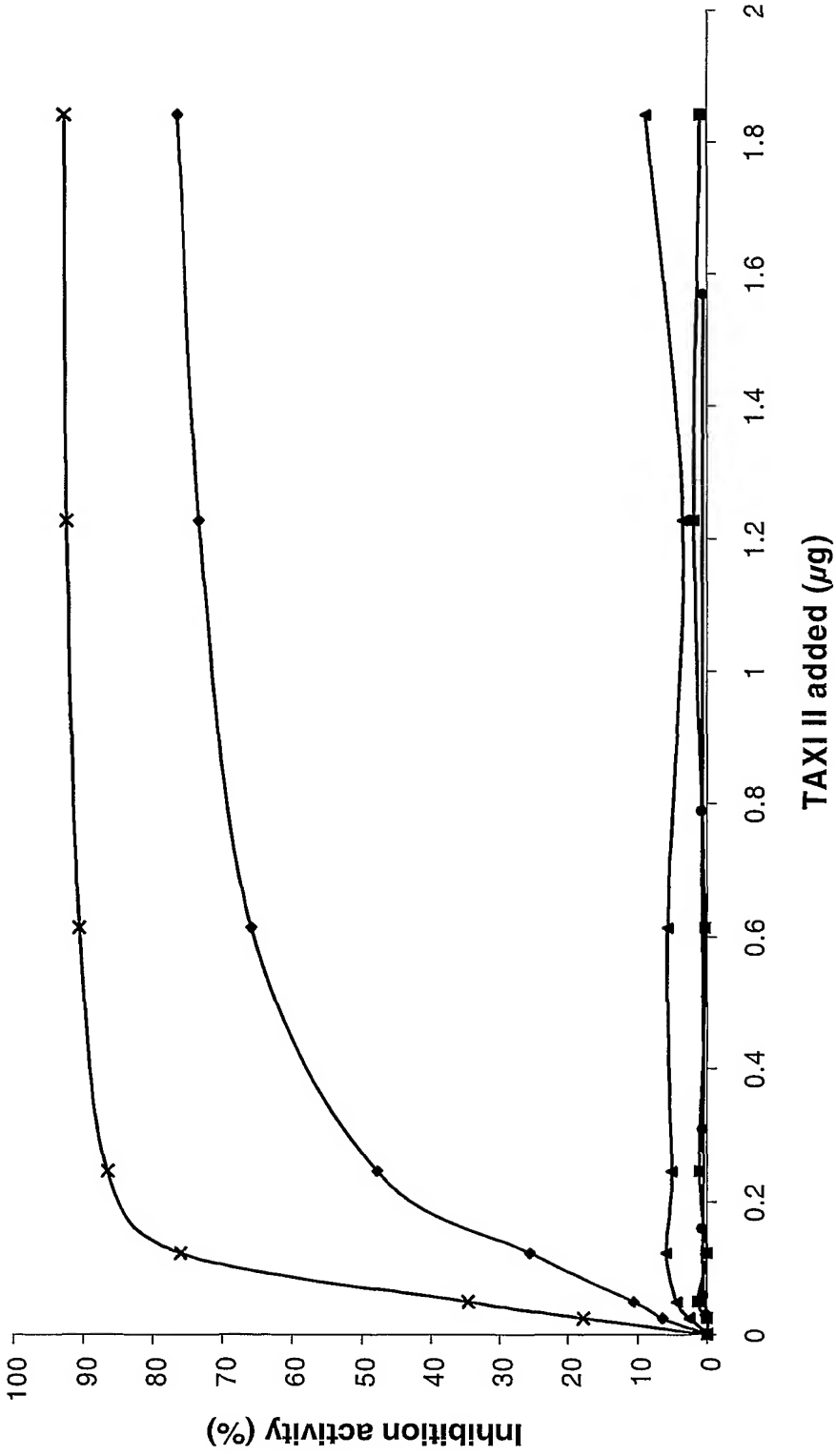
Inhibition activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
TAXI II added (µg)

Figure 5

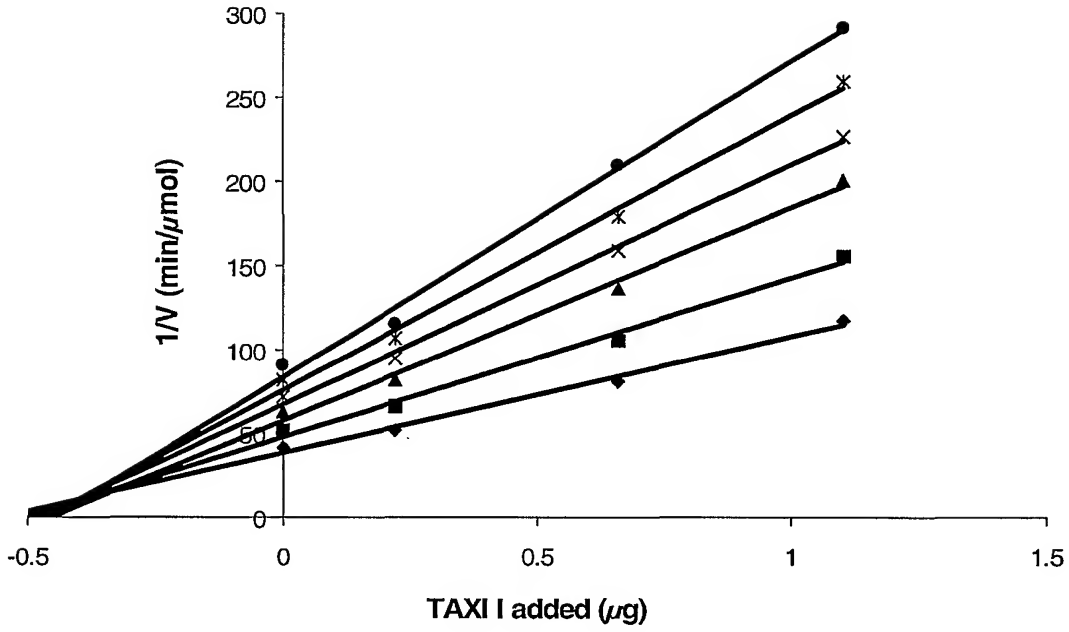

Figure 6

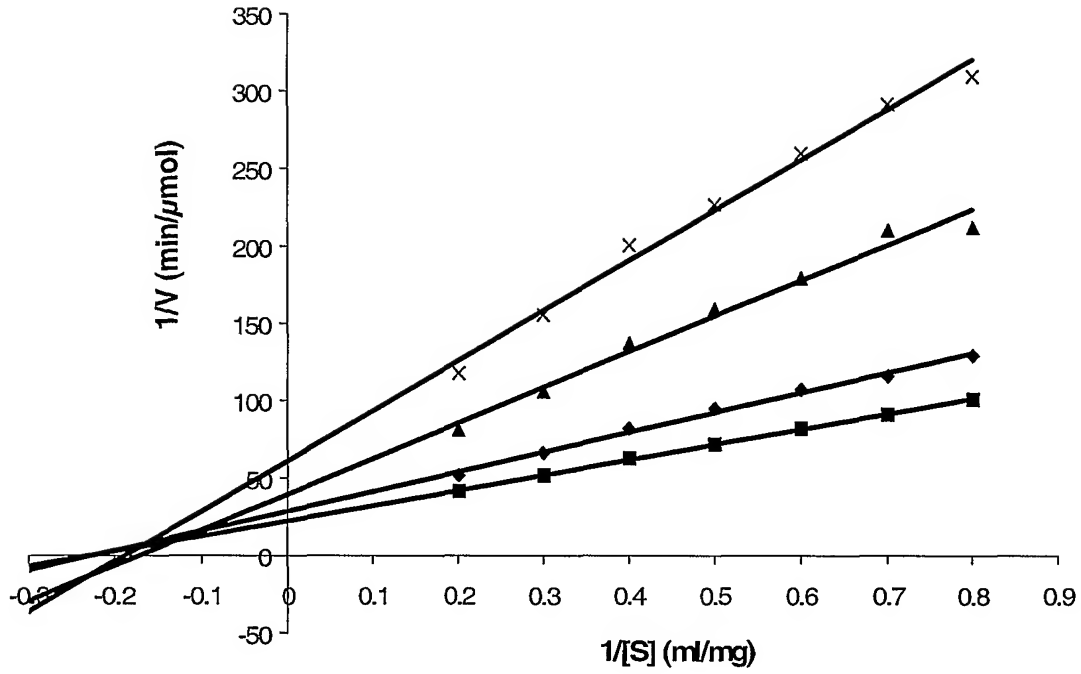
350
300
250
200
150
100
50
0
-50
1/V (min/µmol)
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/[S] (ml/mg)

Figure 7

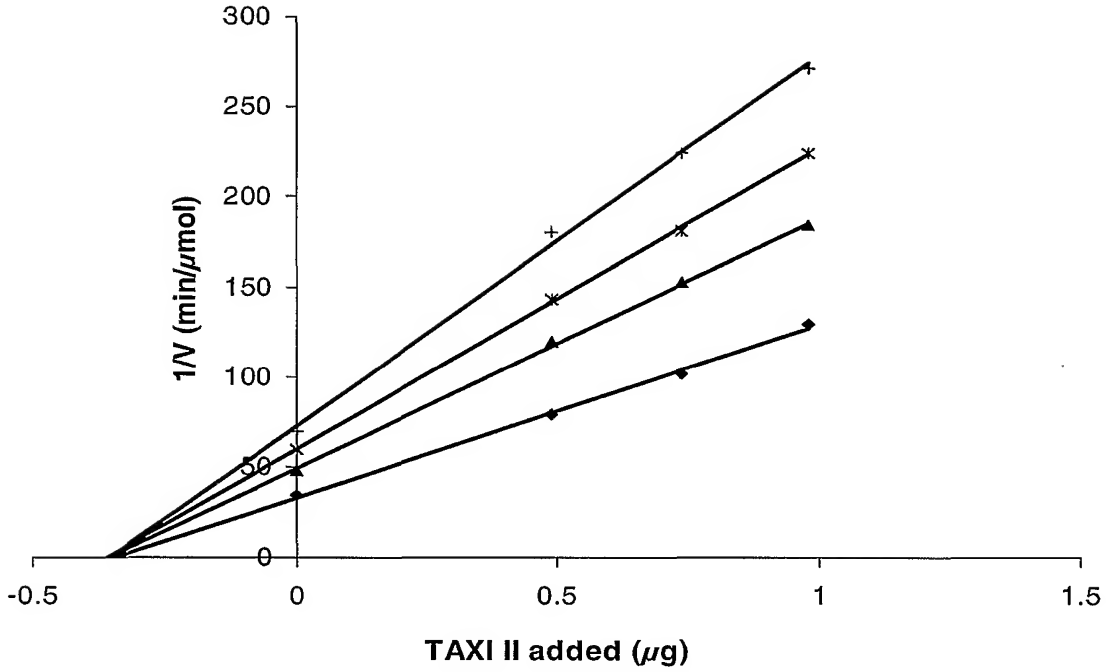

Figure 8

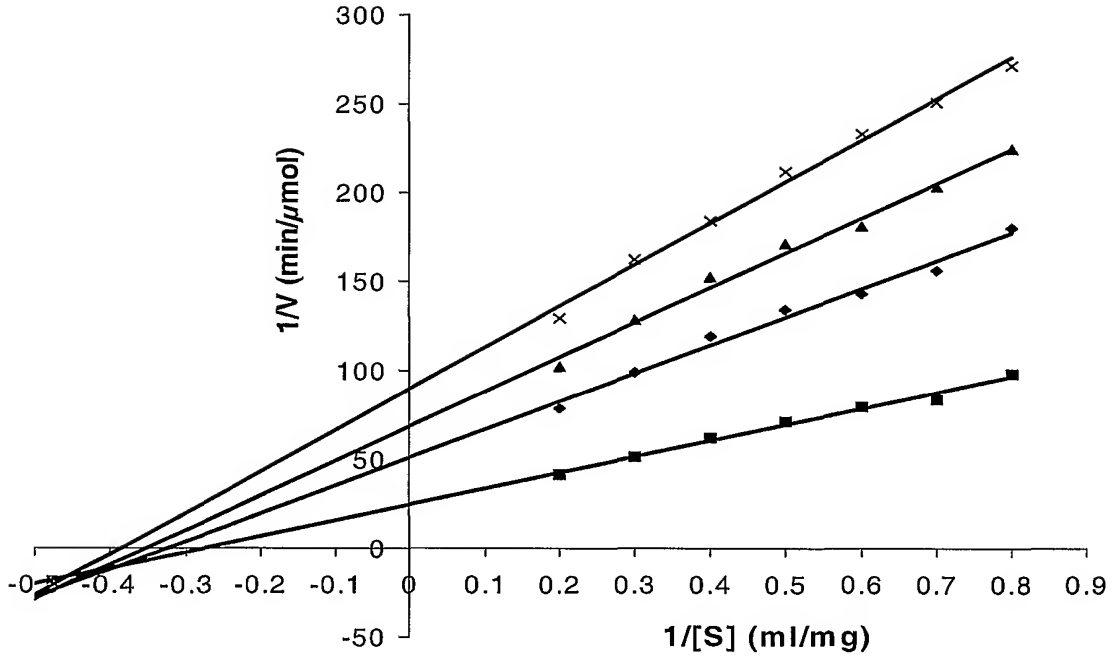
300
250
200
150
100
50
0
-50
1/V (min/µmol)
-0.5
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/[S] (ml/mg)

Figure 9

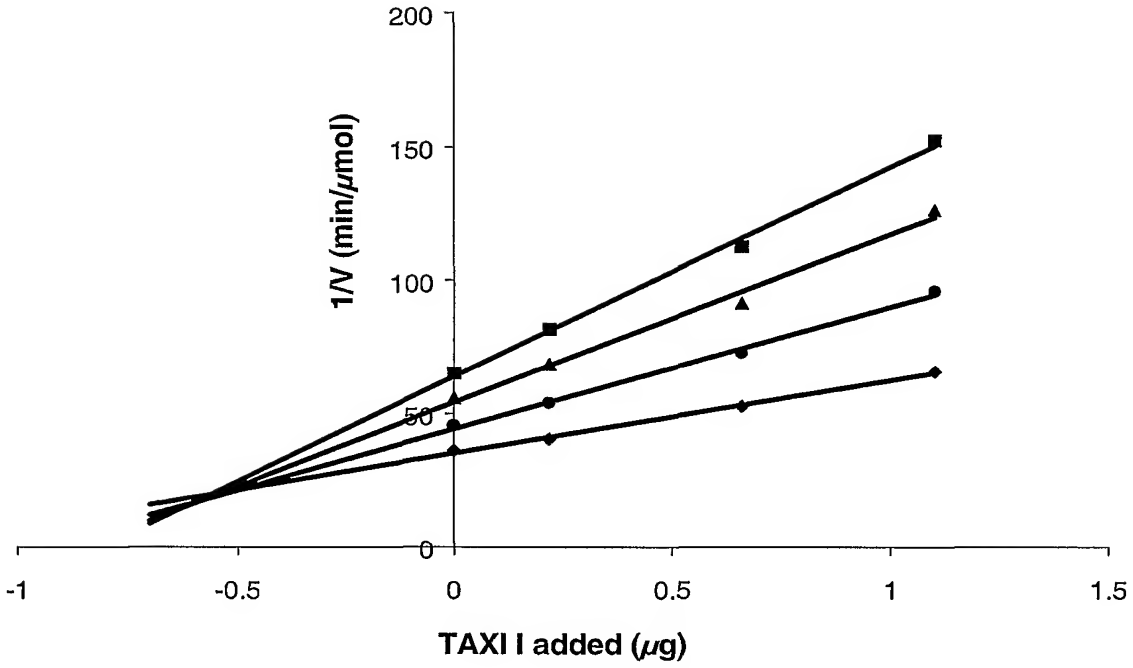

Figure 10

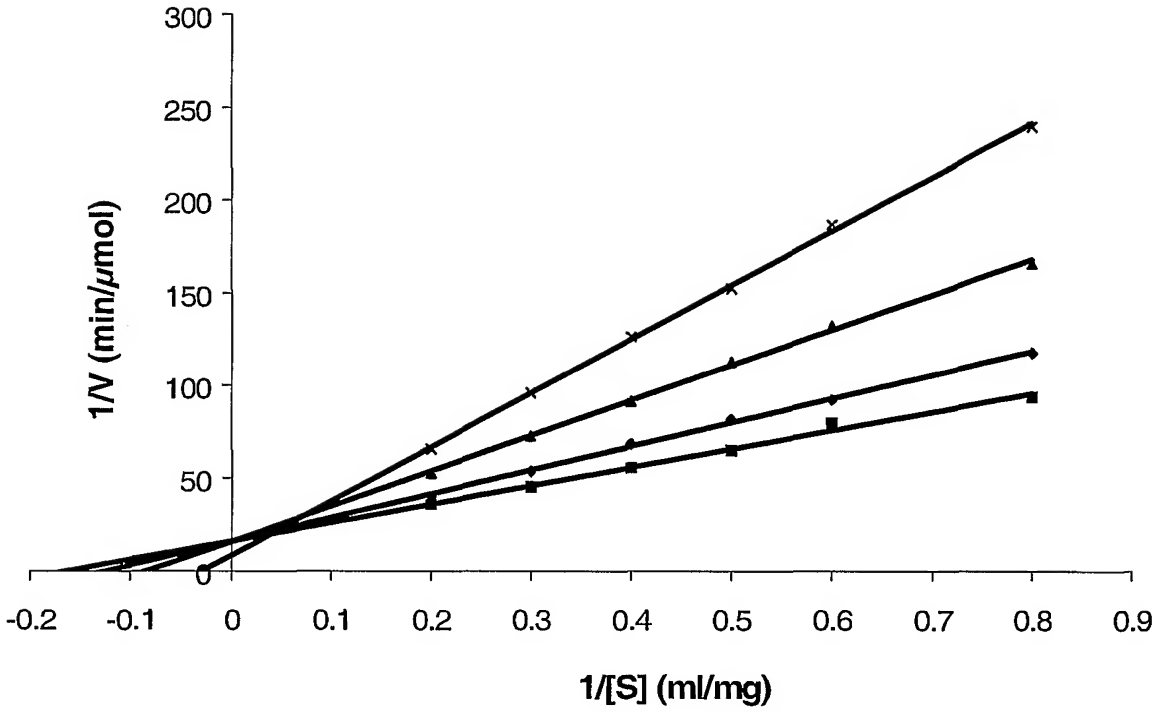
300
250
200
150
100
50
0
1/V (min/µmol)
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1/[S] (ml/mg)

Figure 11

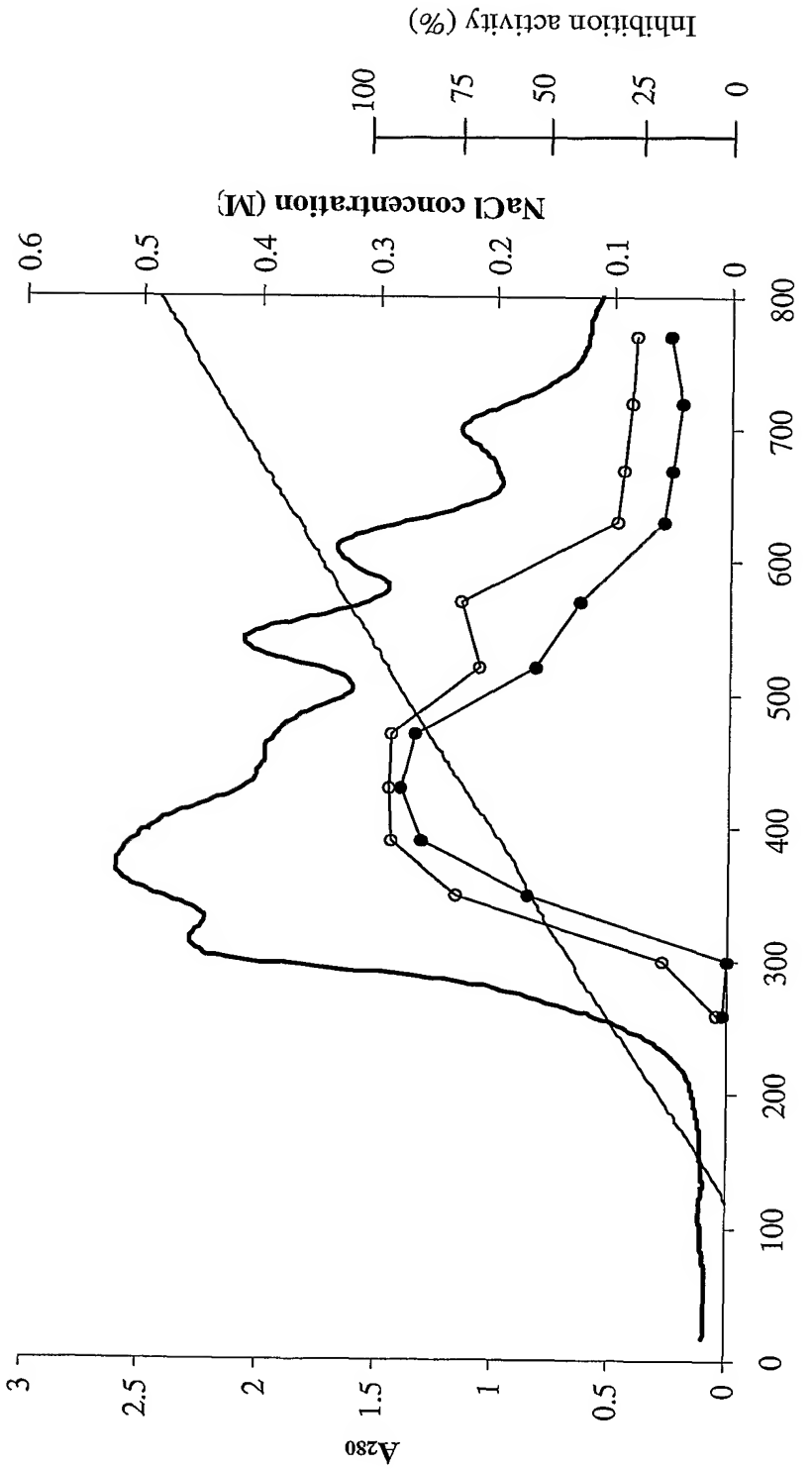
A280
3
2.5
2
1.5
1
0.5
0
0
100
200
300
400
500
600
700
800
Elution volume (mL)
NaCl concentration (M)
0.6
0.5
0.4
0.3
0.2
0.1
0
Inhibition activity (%)
100
75
50
25
0

Figure 12

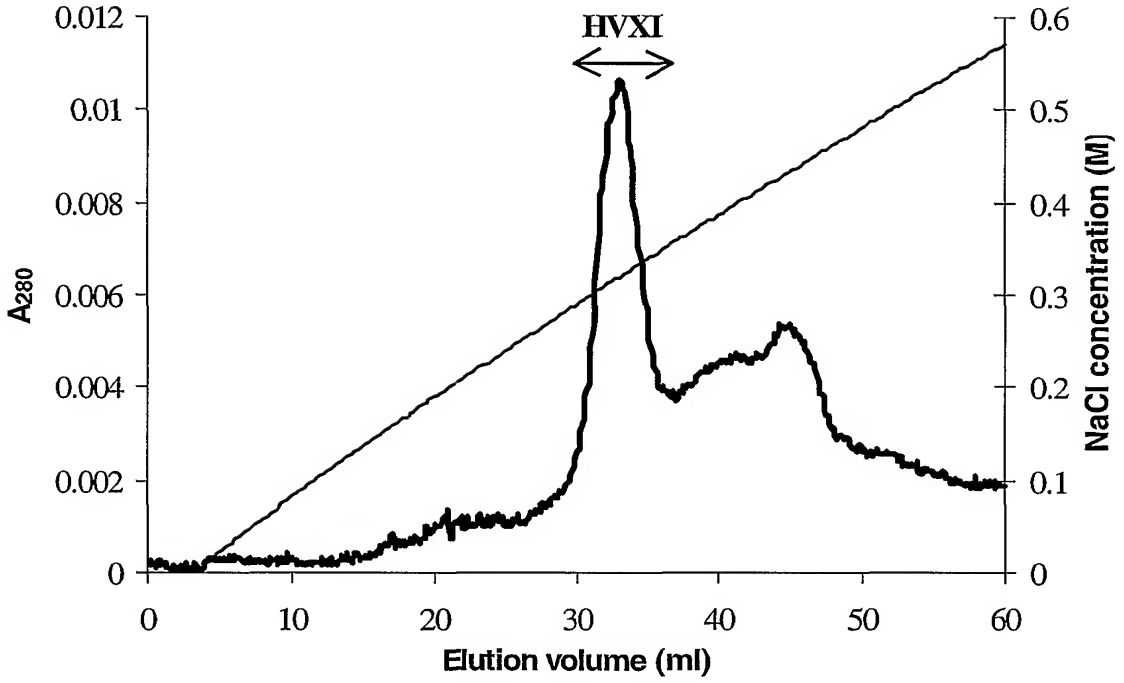
HVXI
0.012
0.01
0.008
0.006
0.004
0.002
0
A280
0.6
0.5
0.4
0.3
0.2
0.1
0
NaCl concentration (M)
0
10
20
30
40
50
60
Elution volume (ml)

Figure 13

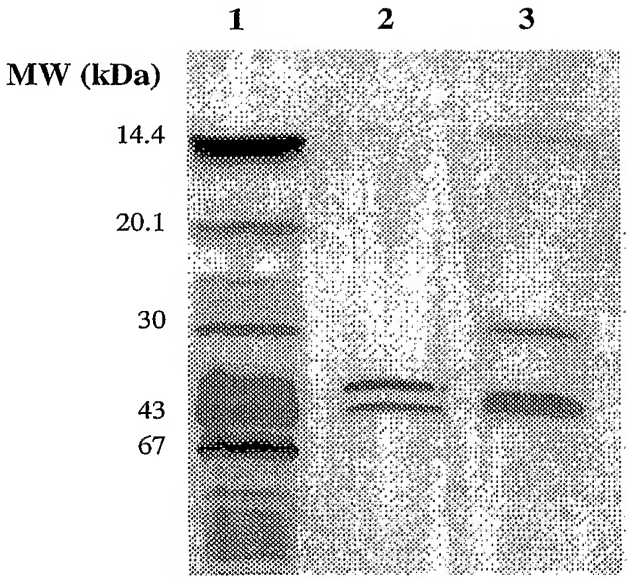

Figure 14

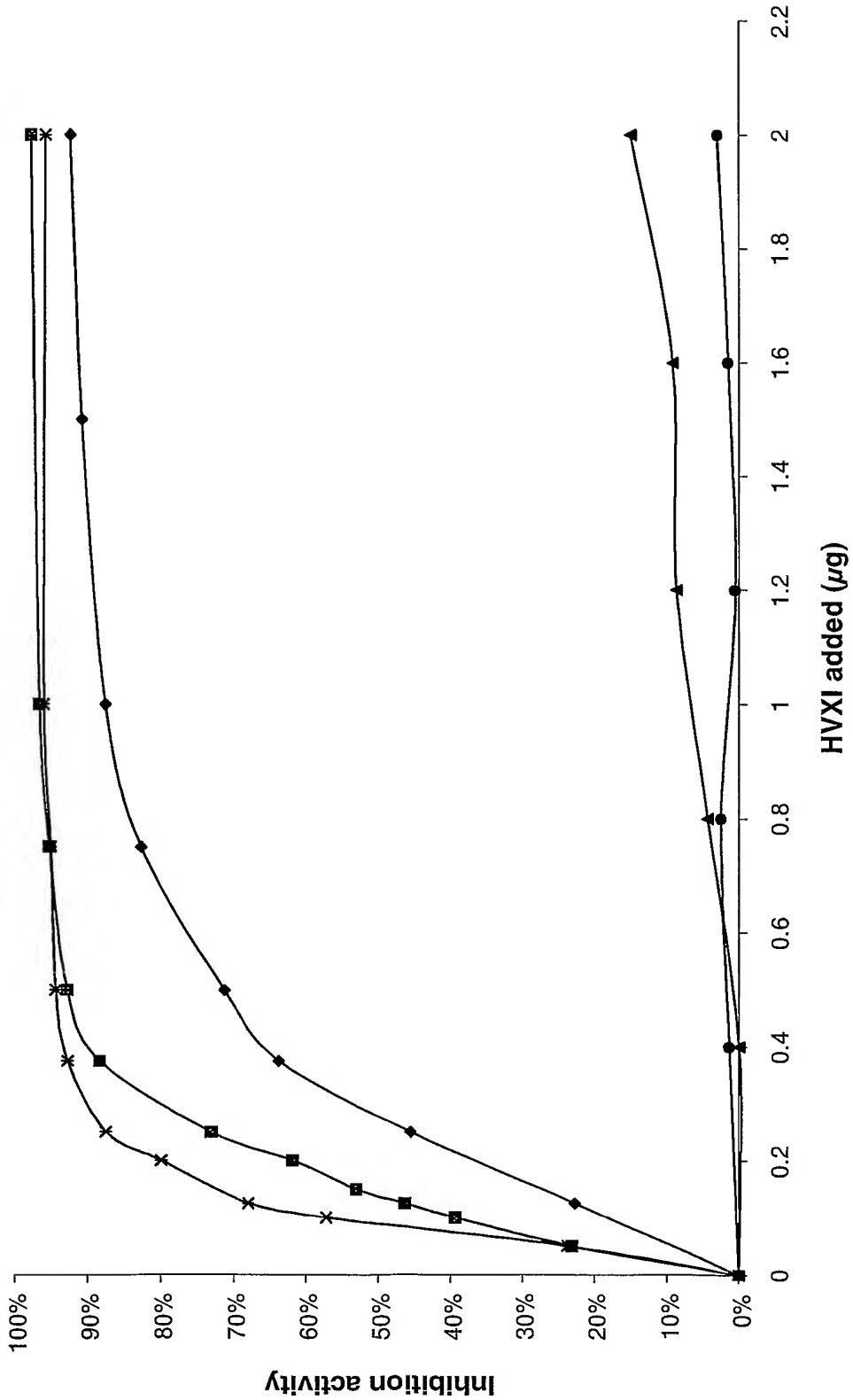
Inhibition activity
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
HVXI added (µg)

Figure 15

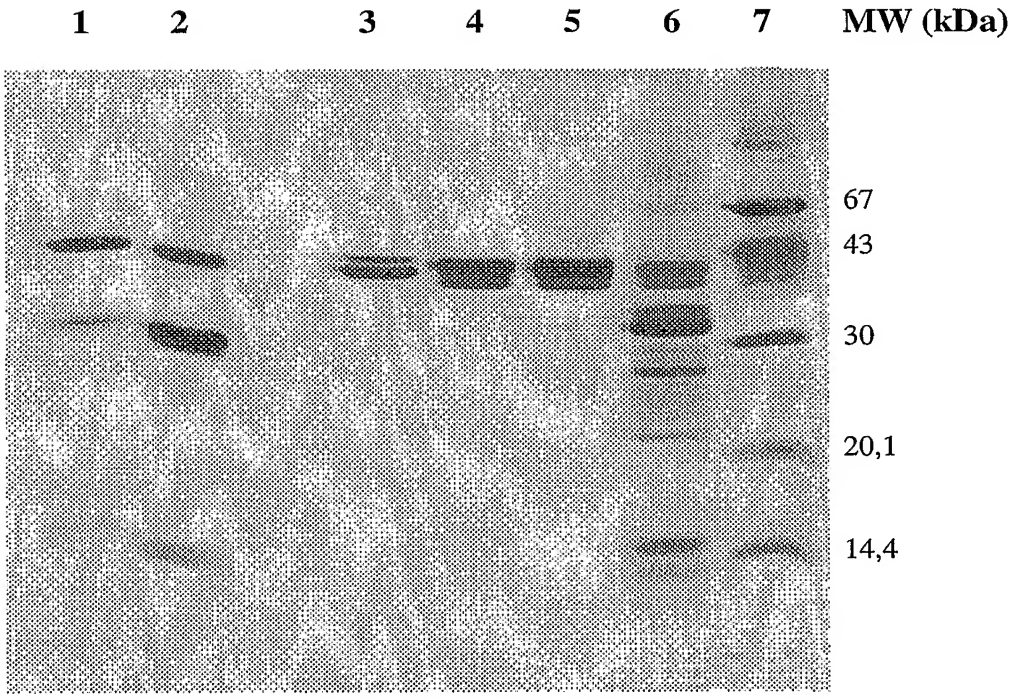

Figure 16

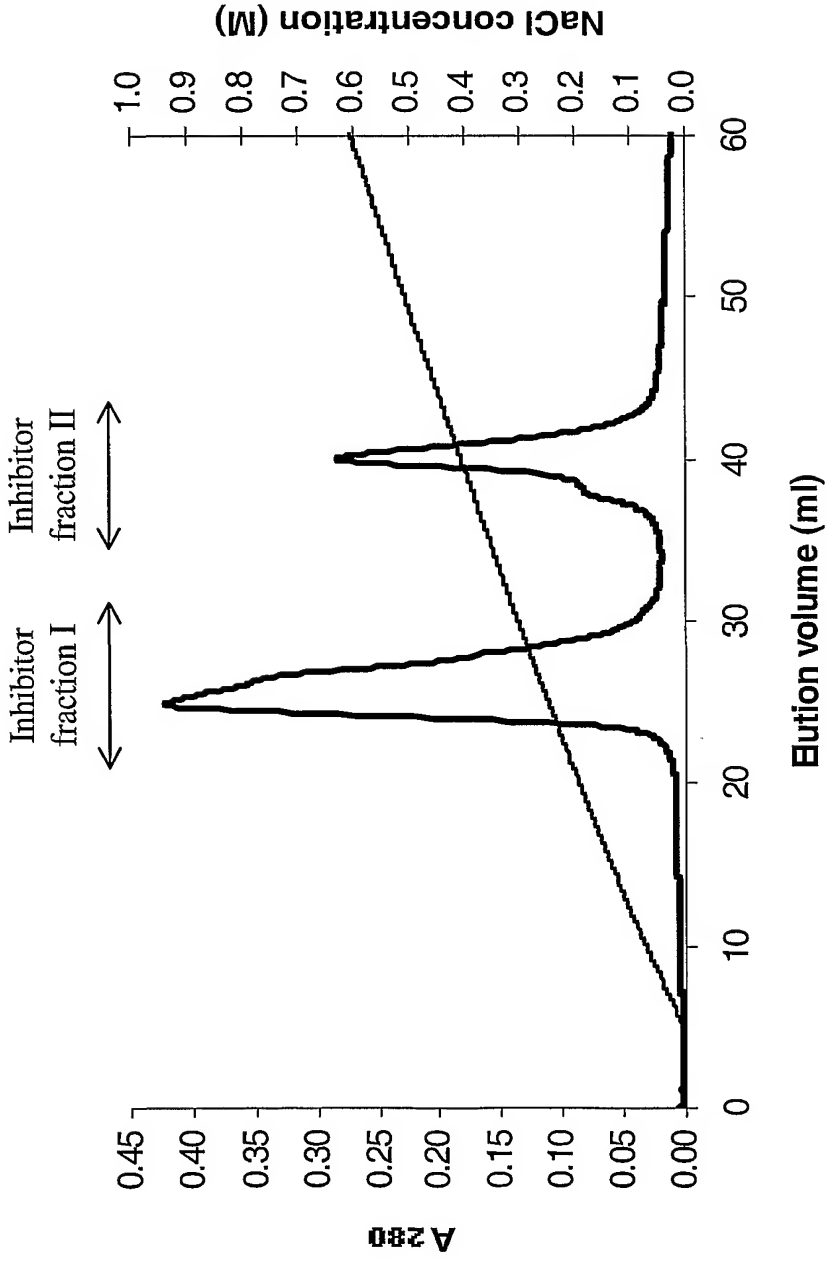
A 280
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
Elution volume (ml)
NaCl concentration (M)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Inhibitor fraction I
Inhibitor fraction II

Figure 17

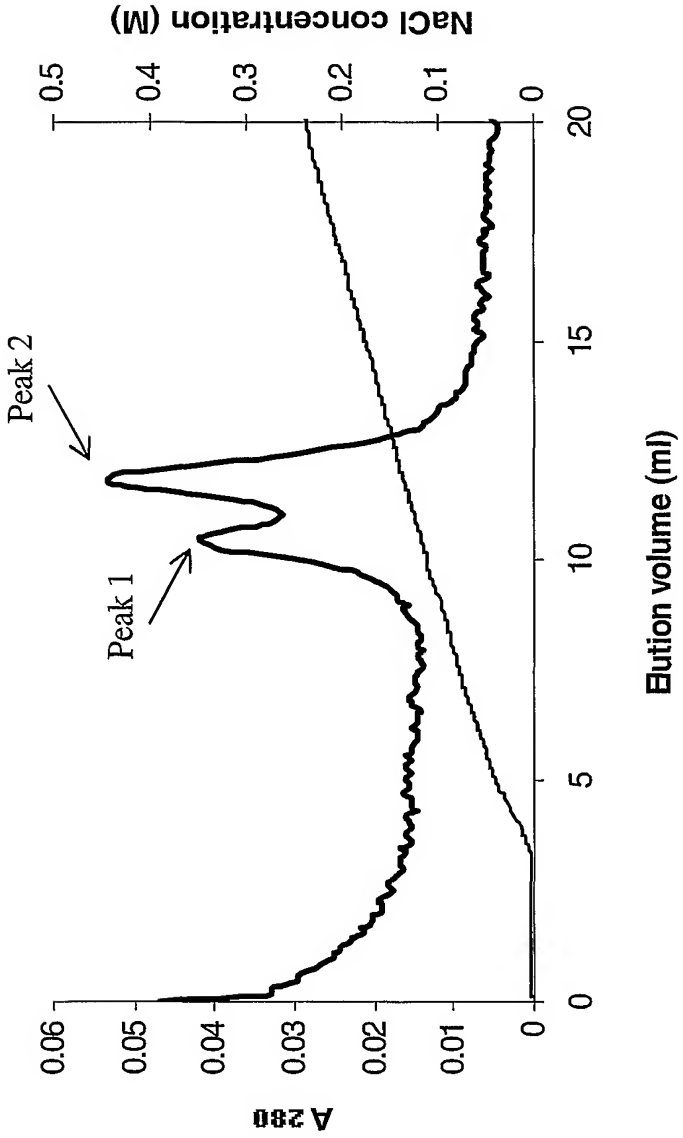
NaCl concentration (M)
0.5
0.4
0.3
0.2
0.1
0
Peak 2
Peak 1
A 280
0.06
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
Elution volume (ml)

Figure 18

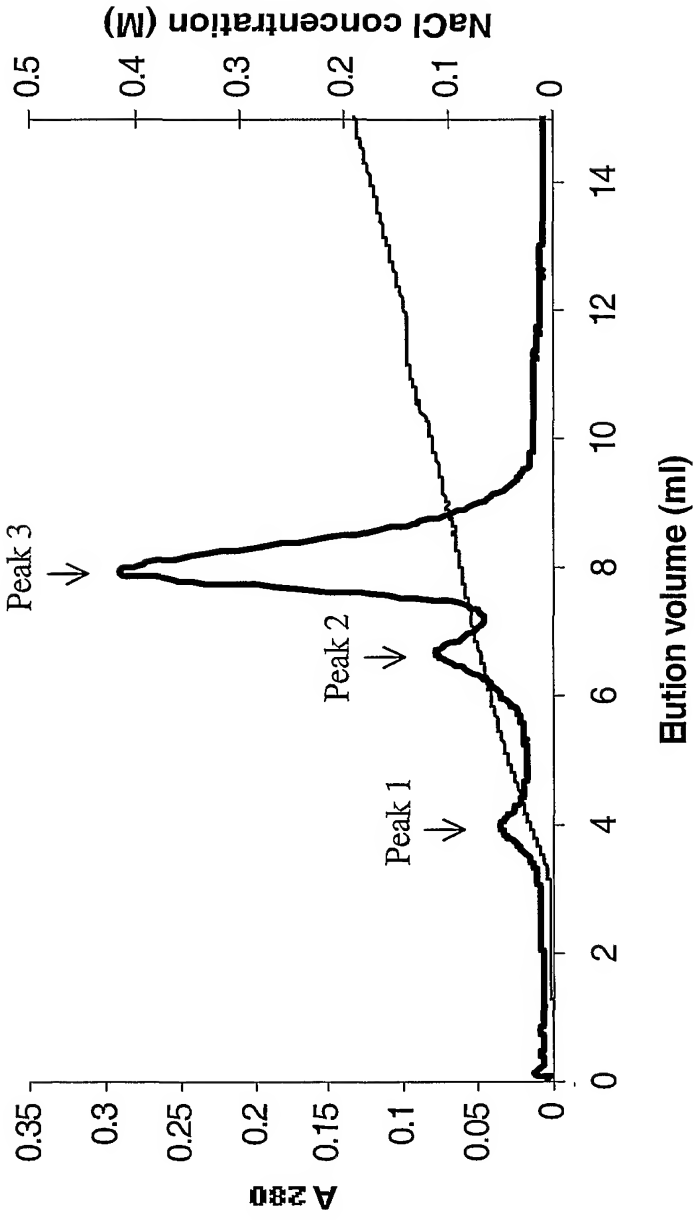
NaCl concentration (M)
0.5
0.4
0.3
0.2
0.1
0
A 280
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
14
Elution volume (ml)
Peak 1
Peak 2
Peak 3

Figure 19

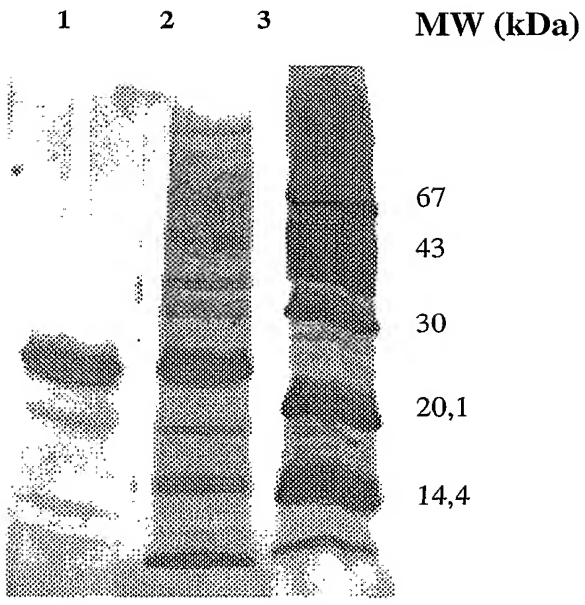

Figure 20

CCA AGA TCT CTG CCA GTT CTG GCA CCT GTG ACC AAA GAT CCA
Pro Arg Ser *Leu Pro Val Leu Ala Pro Val Thr Lys Asp Pro*

TAXI SEQ ID No.21

GCA ACC TCC CTC TAC ACA ATC CCC ..... CTG CCG CA**C TTT**
Ala Thr Ser Leu Tyr Thr Ile Pro Leu Pro His Phe

ACG GGT TGC GGC GGC CTG TAA AGA TCT AGG
Thr Gly Cys Gly Gly Leu Stop Arg Ser Arg

Figure 21

Thioredoxin — Enterokinase recognition site

AAC	CTG	GCC	GGC	TCT	GGA	TCC	GGT	GAT	GAC	GAT	GAC	AAG	CTC
Asn	Leu	Ala	Gly	Ser	Gly	Ser	Gly	Asp	Asp	Asp	Asp	Lys	Leu

PCR product

GCC	CTT	CCA	AGA	TCT	CTG	CCA	.....	CTG	TAA	AGA	TCT	AGG	AAG
Ala	Leu	Pro	Arg	Ser	Leu	Pro		Leu	Stop	Arg	Ser	Arg	Lys

Figure 22

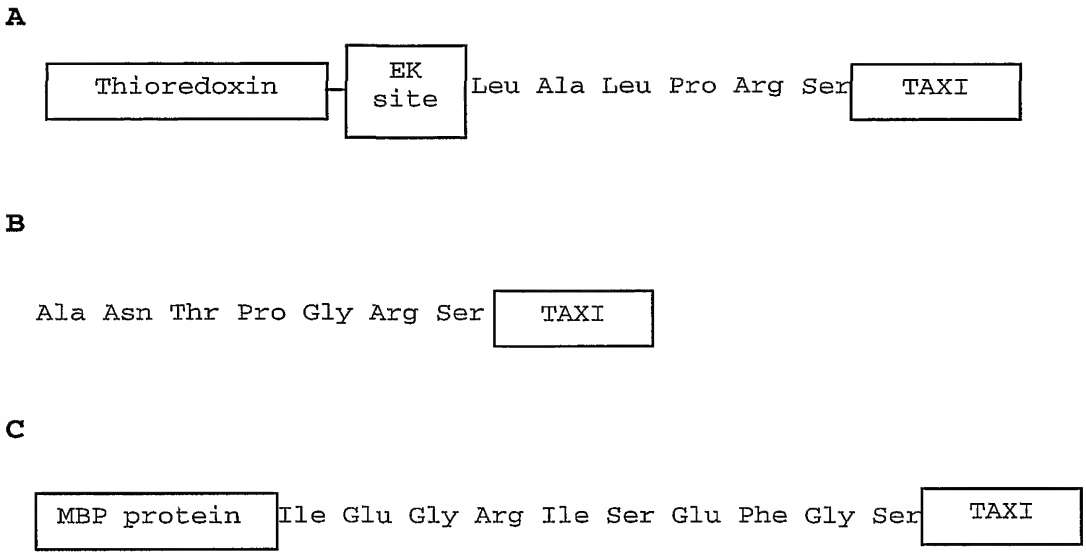
A
Thioredoxin
EK site
Leu Ala Leu Pro Arg Ser
TAXI
B
Ala Asn Thr Pro Gly Arg Ser
TAXI
C
MBP protein
Ile Glu Gly Arg Ile Ser Glu Phe Gly Ser
TAXI

Figure 23

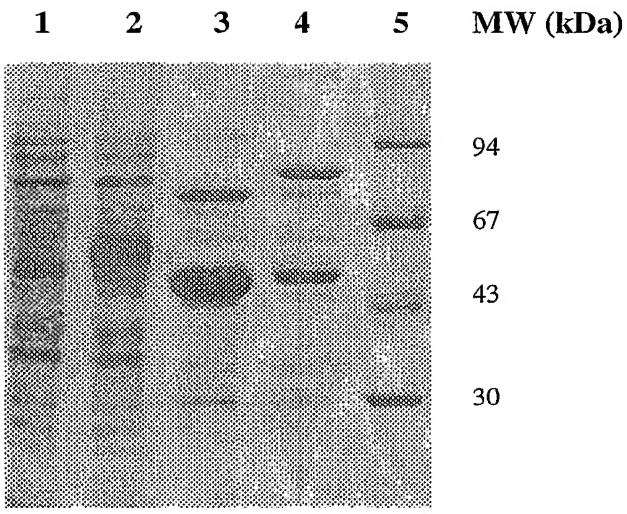

Figure 24

PelB

TTA	TTA	CTC	GCG	GCC	CAG	CCG	GCC	ATG	GCT	**GCC**	**AAC**	**ACA**	**CCC**
Leu	Leu	Leu	Ala	Ala	Gln	Pro	Ala	Met	Ala	**Ala**	**Asn**	**Thr**	**Pro**

PCR product

GGG	AGA	TCT	CTG	CCA	.....	GGC	CTG	TAA	AGA	TCT	TTC	GAA
Gly	Arg	Ser	*Leu*	*Pro*		*Gly*	*Leu*	Stop	Arg	Ser	Phe	Glu

Figure 25

malE Factor Xa recognition site

AAC	AAT	AAC	AAC	AAC	CTC	GGG	ATC	GAG	GGA	AGG	ATT	TCA	GAA
Asn	Asn	Asn	Asn	Asn	Leu	Gly	Ile	Glu	Gly	Arg	Ile	Ser	Glu

PCR product

TTC	GGA	TCT	CTG	CCA	.....		GGC	CTG	TAA	AGA	TCC	TCT	AGA
Phe	Gly	Ser	*Leu*	*Pro*			*Gly*	*Leu*	Stop	Arg	Ser	Ser	Arg

Figure 26

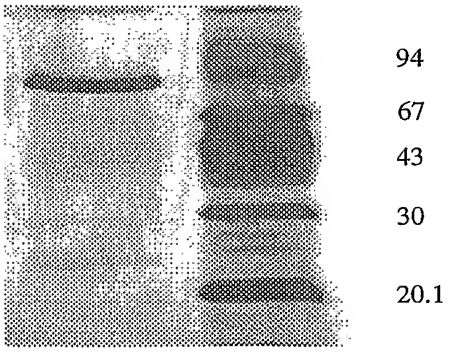

Figure 27

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE 01/00106

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/42 C07K14/415 C07K1/16 C07K16/16 A21D8/00
A23L1/105

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A21D A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
SEQUENCE SEARCH, MEDLINE, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEBYSER W ET AL: "TRITICUM ZESTIVUM XYLANASE INHIBITOR (TAXI), A NEW CLASS OF ENZYME INHIBITOR AFFECTING BREADMAKING PERFORMANCE" JOURNAL OF CEREAL SCIENCE, ACADEMIC PRESS LTD, XX, vol. 30, no. 1, July 1999 (1999-07), pages 39-43, XP000925298 ISSN: 0733-5210 cited in the application the whole document ---	1-161
X	WO 98 49278 A (DEBYSER WINOK ;DELCOUR JAN (BE); LEUVEN K U RES & DEV (BE)) 5 November 1998 (1998-11-05) cited in the application the whole document ---	1-161

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 2001	07/11/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Herrmann, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MCLAUCHLAN W R ET AL: "A NOVEL CLASS OF PROTEIN FROM WHEAT WHICH INHIBITS XYLANASES" BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 338, no. 2, 1 March 1999 (1999-03-01), pages 441-446, XP000925393 ISSN: 0264-6021 the whole document	1-161
X	EP 0 979 830 A (TNO) 16 February 2000 (2000-02-16) the whole document	1-161
P,X	WO 00 39289 A (SOERENSEN JENS FRISBAEK ;DANISCO (DK); SIBBESEN OLE (DK)) 6 July 2000 (2000-07-06) the whole document	1-161
P,X	GEBRUERS KURT ET AL: "Triticum aestivum L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities." BIOCHEMICAL JOURNAL, vol. 353, no. 2, 2001, pages 239-244, XP001022202 ISSN: 0264-6021 the whole document	1-161
T	GOESAERT H ET AL: "Purification and partial characterization of an endoxylanase inhibitor from barley." CEREAL CHEMISTRY, vol. 78, no. 4, July 2001 (2001-07), pages 453-457, XP001033832 ISSN: 0009-0352 the whole document	1-161

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-161 (all partially)

Support, disclosure, clarity:
Present set of claims relates to products and methods defined by reference to a desirable characteristic or property, namely inhibitors of cellulose, xylan, arabinoxylan or beta-glucan degrading enzymes. The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Art. 6 PCT and/or disclosure within the meaning of Art. 5 PCT for only inhibitors of "endoxylanases" (see p. 16, l. 3-8 of present description). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Art. 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Clarity, conciseness:
In view of the large number and also the wording of the claims presently on file, which render it difficult, if not impossible, to determine the matter for which protection is sought, the present application fails to comply with the clarity and conciseness requirements of Art. 6 PCT (see also Rule 6.1(a) PCT) to such an extent that a meaningful search is impossible.

Clarity, conciseness:
Present set of claims relates to an extremely large number of possible endoxylanase inhibitors and methods for the isolation of such peptides. In fact, the claims contain so many options, variables, possible permutations and provisos that a lack of clarity and conciseness within the meaning of Art. 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Besides TAXI I, TAXI II and HvXI numerous other endoxylanse inhibitors and variants, homologues and fragments thereof are claimed.

CONSEQUENTLY, THE SEARCH HAS BEEN CARRIED OUT FOR THE GENERAL ASPECT OF THE INVENTION, NAMELY "PROTEINS WHICH INHIBIT ENDOXYLANASES".

Unity:
According to Art. 3(4)(iii) and Rule 13 PCT an application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept, i.e., having at least one common technical feature defining a contribution over the prior art.

The original common concept which might have linked the above mentioned inventions is the provision of endoxylanase inhibitors. This common concept is however not novel with regard to prior art: The documents mentioned in this search report all disclose proteins which inhibit endoxylanases.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Since no other technical feature can be distinguished which might link the subject-matter of present claims, each single endoxylanse inhibitor and each method for the separation or isolation of such inhibitors claimed represents an independent invention. Due to the reasons given above (lack of clarity and conciseness) it is however impossible to determine the exact number of individual endoxylanase inhibitors claimed.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

'ormation on patent family members

Ir onal Application No
PCT/BE 01/00106

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9849278	A	05-11-1998	AU	7761198 A	24-11-1998
			BR	9809348 A	04-07-2000
			CN	1254374 T	24-05-2000
			WO	9849278 A1	05-11-1998
			EP	0996709 A1	03-05-2000
EP 0979830	A	16-02-2000	EP	0979830 A1	16-02-2000
WO 0039289	A	06-07-2000	AU	1676600 A	31-07-2000
			BR	9916507 A	02-10-2001
			EP	1141254 A1	10-10-2001
			FR	2788781 A1	28-07-2000
			WO	0039289 A2	06-07-2000